

Quantitation of folates and their maillard products in foods by stable isotope dilution assays

Quantifizierung von Folaten und ihren Maillard-Produkten in Lebensmitteln durch Stabilisotopenverdünnungsanalysen

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Summary

The recently developed stable isotope dilution assay (SIDA) for folates was used for the quantitation of folic acid in fortified foods.

The results revealed that the labelled contents were partly exceeded by about 30 %, and partly the contents were significantly lower than the label claim. Therefore, the manufacturers are called upon adjusting the folate contents more accurately, whereas the official laboratories should control the contents more frequently.

In further experiments, the nonenzymatic glycation product of folic acid, N²-[1-(carboxy)ethyl]folic acid (CEF), was identified in baked foods made from fortified flour. Application of a SIDA to commercial cookies produced from wheat flour fortified with folic acid revealed CEF contents of up to 7.1 µg/100 g, which correlated to about 10 to 20 % of the cookies' folic acid content. Therefore, to retain a maximum amount of folic acid, fortified products should rather be baked with sucrose than with reducing carbohydrates.

Foods being heated for preservation while containing high amounts of endogenous folates and sugars were assessed for carboxyethylated folates. None of these compounds were found neither in model systems nor in foods such as strawberry jams.

Keywords:

carboxyethylfolic acid, folic acid, liquid chromatography – tandem mass spectrometry, non-enzymatic glycation, stable isotope dilution assay

Zusammenfassung

Die kürzlich entwickelte Stabilisotopenverdünnungsanalyse (SIVA) für Folate wurde eingesetzt, um Folsäure in vitaminangereicherten Produkten zu quantifizieren.

Die Ergebnisse zeigten, dass die gekennzeichneten Gehalte teils um 30 % überschritten wurden, die Folatgehalte teilweise aber auch deutlich unter den Packungsangaben lagen. Daher muss von den Herstellern eine verlässlichere Einstellung der Folsäuregehalte und von den amtlichen Untersuchungslabors eine häufigere Kontrolle derselben gefordert werden.

In weiteren Versuchen wurde ein Reaktionsprodukt von Folsäure mit reduzierenden Kohlenhydraten, N²-[1-(Carboxy)ethyl]folsäure (CEF), in aus angereichertem Mehl hergestellten Backwaren nachgewiesen. Die mit Hilfe einer SIVA durchgeführte Quantifizierung ergab in Keksen, die mit folsäureangereichertem Mehl gebacken wurden, CEF-Gehalte von bis zu 7.1 µg/100 g, was 10 bis 20 % des Folsäuregehaltes der Kekse entsprach. Um einen möglichst niedrigen Verlust an Folsäure in solchen Produkten zu gewährleisten, sollten diese eher mit Saccharose als mit reduzierenden Kohlenhydraten gebacken werden.

Zudem wurden Lebensmittel mit hohem natürlichen Folatgehalt, die in Gegenwart von Zuckern zur Konservierung erhitzt worden waren, auf ihre Gehalte an carboxyethylierten Folaten untersucht. Dabei wurden weder in Modellreaktionen noch in Lebensmitteln, wie z. B. Erdbeerkonfitüre, Carboxyethyllderivate endogener Folate nachgewiesen.

Kennwörter:

Carboxyethylfolsäure, Folsäure, Flüssigchromatographie – Tandemmassenspektrometrie, Maillardreaktion, Stabilisotopenverdünnungsanalyse

Introduction

With growing evidence of the role of folates in physiology, increased emphasis in nutrition sciences is now placed on these vitamins. In particular the folates are supposed to prevent neural tube defects [1], Alzheimer's disease [2] and cardio vascular disease [3]. Furthermore, the role of folate deficiency in inducing single strand breaks of DNA [4] and in favoring the activation of proto-oncogenes [5] has been recently highlighted. These effects appear to be the cause for

the meanwhile evident correlation between a low dietary intake of folates and the risk of cancer [6].

This knowledge has led to the development of foods supplemented with folic acid (FA) and spurred mandatory fortification with FA in several countries, e. g. in the USA. There, FA is added to wheat flour and becomes a component of pastries as well as of other cereal-containing foods. As FA undergoes thermal treatment in this way, its reactions with other ingredients in foods have to be considered.

In this context, a nonenzymatic glycation involving carbohydrates and FA acting as an amino compound has been reported recently [7]. The product, N²-[1-(1-carboxy)ethyl]folic acid (CEF), has been structurally characterized and its formation has been followed in models involving different carbohydrates and their degradation products. As sugar products such as corn syrup or high fructose corn syrup (HFCS) are common sweeteners, the question arises about the occurrence of CEF in fortified foods. Further carboxyethylated derivatives of endogenously occurring folates in foods, have not been reported yet.

As we recently reported on the analysis of endogenous folates in foods [8], the use of stable isotopomers of folate vitamers enables to correct for losses during extraction, clean-up, HPLC and MS detection. The purpose of the present study was, therefore, first to quantitate FA in commercial products by using the already existing stable isotope dilution assay (SIDA) and compare the resulting data with the labelled folate contents. Secondly, a sensitive and accurate method for CEF quantification was to develop and products fortified with FA were to screen for their content of CEF. Moreover, the third aim of the study was to elucidate, whether endogenously occurring folates also can be carboxyethylated and whether these products are detectable in foods high in folates.

Experimental

Chemicals

The following chemicals were obtained commercially from the sources given in parentheses: acetonitrile, dihydroxyacetone, folic acid, formic acid, 2-mercapto ethanol, methanol, NaHCO₃, KH₂PO₄, Na₂HPO₄, sodium acetate, sodium chloride (Merck, Darmstadt, Germany) CHES, HEPES, sodium ascorbate (Sigma, Deisenhofen, Germany).

Extraction buffer consisted of aqueous HEPES (50 mmol/L) and aqueous CHES (50 mmol/L) at pH 7.85 and contained sodium ascorbate (2 %) and 2-mercapto ethanol (20 mM).

[²H₄]-folic acid [9], CEF and [²H₄]-CEF [10] were synthesized as reported recently.

Commercial food samples

Multivitamin juices, whey products, breakfast cereals, sweets, strawberry jams, baby foods and further fortified foods except cookies were obtained from local retail stores in the city of Munich, Germany. Six different types of commercial cookies were purchased at supermarkets in Washington, DC, USA.

Model reactions for generation of glycation products of endogenous folates

5-methyltetrahydrofolic acid (0.5 mmol) was reacted

with dihydroxyacetone (2 mmol) in phosphate buffer (1 mol/L, pH 7.4) at 100 °C for 24 h. The reaction mixture was analysed by LC-MS and LC-MS/MS for N²-[1-(carboxy)ethyl]-5-methyltetrahydrofolic acid in the positive ESI mode scanning for m/z 532 and the MS/MS transition 532 → 487 at collision energies ranging from 15 to 25 %, respectively.

Extraction of folates and glycated folates in foods

Solid samples were frozen in liquid nitrogen and minced in a blender (Privileg, Quelle, Fürth, Germany). The resulting powders or liquid samples (1 g) were extracted as detailed recently [10].

Sample Clean-up by Solid Phase Extraction (SPE)

Extracts were purified by anion exchange SPE according to the method described by *Gounelle et al.* [11]. After applying the sample extracts (6 mL), the columns were washed with six volumes of conditioning buffer, and the folates were eluted with 3 mL of aqueous sodium chloride (5 %, containing 1 % sodium ascorbate and 0.1 mol/L sodium acetate). 100 µL mercapto ethanol was added to each eluate and the purified extracts were subjected to LC-MS/MS.

LC-MS/MS

The samples (20 µL) were injected on a high performance liquid chromatograph equipped with a Nucleosil C-18 reversed phase column (250 x 3 mm; 5 µm, Macherey-Nagel, Germany) that was connected to a Surveyor diode array detector and a TSQ triple quadrupole mass spectrometer (Thermo Science, Bremen, Germany). Gradient elution and mass spectrometry were performed as detailed recently [10].

Determination of response factors for LC-MS/MS

Solutions of FA / [²H₄]-FA and CEF / [²H₄]-CEF in extraction buffer (10 mL) were mixed in five mass ratios ranging from 0.2 to 5 to give total contents of FA and CEF of 2 µg, respectively. Subsequently, the FA and CEF mixtures were subjected to LC-MS/MS as outlined before. Response factors R_i were calculated as reported recently [12].

Results and discussion

Development of stable isotope dilution assays

Quantitation of folic acid

We recently reported on a SIDA to analyze folates by LC-tandem MS [8] based on the use of isotopomeric vitamins as the internal standards.

In the present study, sample preparation for foods fortified with FA proved to be more simple than for those containing endogenous folates. After stirring

the powdered samples for 1 h in extraction buffer containing known amounts of [$^2\text{H}_4$]-folic acid at pH 5.7, the extracts had only to be filtered and passed through a membrane filter. In contrast to non-fortified foods, most of the samples analyzed here contained only minute amounts of conjugated vitamins. Therefore, enzymatic liberation of bound FA by amylase, proteinase, and deconjugase [8] was evitable.

Quantitation of CEF

Schneider et al. [7] recently found a glycation product of FA; CEF in models containing sugars and FA, CEF is a conceivable compound in fortified foods that are similarly composed and undergo a similar treatment like the models used by the latter authors. Possible examples are multivitamin juices containing carbohydrates and being pasteurized for becoming shelf-stable. Furthermore, cookies produced from folate-fortified flour and glucose or fructose syrup may contain CEF. Therefore, we decided to develop a SIDA also for this folate derivative.

Synthesis of N^2 -[1-(1-carboxy)ethyl]folic acid

As CEF is not commercially available, initial experiments were run to synthesize it as a reference compound. The most successful way was the one described by *Schneider* et al. [7] by reacting FA with dihydroxyacetone (DHA) and purifying CEF from unreacted FA by preparative HPLC [10]. NMR experiments revealed similar data as reported by the latter authors.

LC-MS properties of CEF

In acidic eluents, folates form protonated molecules dissociate upon MS/MS by loss of glutamic acid [9]. Expectedly, CEF behaved similarly upon collision-induced dissociation (CID). However, the intensity in LC-MS and LC-MS/MS was one order of magnitude lower than that of folic acid, thus indicating that carboxyethylation of the exocyclic amino moiety lowers decisively the ionization yield.

Synthesis of labelled CEF

During the development of a SIDA for folates, we had been starting with the synthesis of deuterated FA to generate the [$^2\text{H}_4$]-labelled isotopomers of the most important folate vitamers that occur endogenously in foods [9]. Therefore, it appeared straightforward to synthesize labeled CEF by starting from [$^2\text{H}_4$]-FA, too. Synthesis of [$^2\text{H}_4$]-CEF was performed analogously to unlabelled CEF by reacting [$^2\text{H}_4$]-FA with DHA and subsequent purification by preparative HPLC as described by *Rychlik and Mayr* [10]. Briefly, aliquots of the raw synthesis were separated using a Hyperclone ODS column (5 μm , 250 x 10 mm, Phenomenex, Aschaffenburg, Germany) coupled to an HPLC system (Biotek, Eching, Germany) and eluted with a linear

gradient starting from a mixture of methanol in aqueous formic acid (0.1 %; 12+88, v+v) to 100 % methanol at 17 min at a flow rate of 2.5 mL/min. [$^2\text{H}_4$]-CEF was eluted as the highest peak at 280 nm and was collected from ten runs.

Development of a stable isotope dilution assay for CEF

To convert the measured ion intensities into the mass ratios of labelled and unlabelled CEF, a graph was calculated from calibration mixtures of known mass ratios and the corresponding peak area ratios in LC-MS/MS. Good response linearity was demonstrated for mass ratios ranging from 0.2 to 5 [10].

As the CEF content in foods was expected to be quite low, sensitivity of LC-MS was evaluated by determining the detection limit (DL) in edible starch according to the method of *Hädrich and Vogelgesang* [13]. The calculations resulted in a DL of 0.4 $\mu\text{g}/100\text{ g}$ and a quantification limit of 1.3 $\mu\text{g}/100\text{ g}$ in cereal-based foods.

Fortified Samples	Folic acid mg/100 g		FA, % of claim	CEF $\mu\text{g}/100\text{ g}$
	analyzed	Label claim		
Fruit juices	0.24	0.20	120	n.d.
	0.07	0.07	103	n.d.
	0.12	0.10	116	n.d.
	0.09	0.10	94	n.d.
Whey products	0.04	0.03	133	n.d.
	0.05	0.04	131	n.d.
Breakfast Cereals	0.27	0.17	161	n.d.
	0.43	0.20	213	n.d.
Sweets	0.70	0.60	117	n.d.
	0.57	0.80	72	n.d.
Wheat flour	0.14	0.14	103	n.d.
Margarine	1.26	1.00	126	n.d.
Salt	8.38	10.0	84	n.d.
Baby food	0.11	0.17	64 ^a	n.d.
	0.05	0.07	72 ^a	n.d.
	0.14	0.14	99 ^a	n.d.
	0.05	0.08	61 ^a	n.d.
Cookies, made from fortified flour	0.04	-	-	5.1
	0.03	-	-	7.1
	0.10	-	-	6.8
Cookies, sugar-free, made from fortified flour	0.06	-	-	n.d.
	0.07	-	-	n.d.
Cookies, made from non-fortified flour	0.0004	-	-	n.d.

Tab. 1: Analyzed and labelled contents of folic acid (FA) and N^2 -[1-(1-carboxyethyl)]folic acid (CEF) in foods

n.d. not detectable

^a below 100 % due to endogenous food folates, which were not quantified in this study

To avoid interferences in LC-MS/MS detection, we used anion exchange chromatography (AEC) for purification, which provided extracts with less background noise than without sample clean-up. Interestingly, the intensity ratio between FA and CEF after AEC was very similar to that without using this clean-up, thus indicating that CEF was not discriminated when binding to the anion exchange material.

Results of the quantifications of foods

Several foods fortified with several vitamins were surveyed to prove the suitability of the new method. Of liquid products, four fortified fruit juices and two whey products were quantified. Moreover, two samples of breakfast cereals, two sweets and six cookies were analyzed (Fig. 1). Finally we quantified four baby foods as well as a wheat flour, margarine, and salt, the latter of those were solely fortified with folic acid. The results of the quantifications are presented in Table 1. Considering folic acid, the contents ranged between 30 and 8380 µg/100 g and exceeded for eight products

the label claim, whereas two products contained significantly lower amounts than labelled. Interestingly, all four baby foods contained less folic acid than claimed on the labels. However, as milk products were the main ingredients of all formulas, significant amounts of other folate vitamers, e. g. 5-methyltetrahydrofolate, have to be expected. Therefore, the total folate content may be in agreement with the data given on the labels for folic acid. Only two fruit juices and the wheat flour were well in line with the label within a tolerance of 10 %. Remarkable discrepancies were found in case of the breakfast cereals, which exceeded the label claim by more than 50 %. Although an over dosage is thought to be reasonable to anticipate losses during manufacture and storage, these differences to the label appear too high. These findings are quite in good accordance with the results of Osseyi et al. [14], who analyzed folic acid in fortified cereal products by LC-UV. However, the data of the latter authors ranged between 72 and 147 % of the label claim and thus showed lower discrepancies than the products analyzed in the present study.

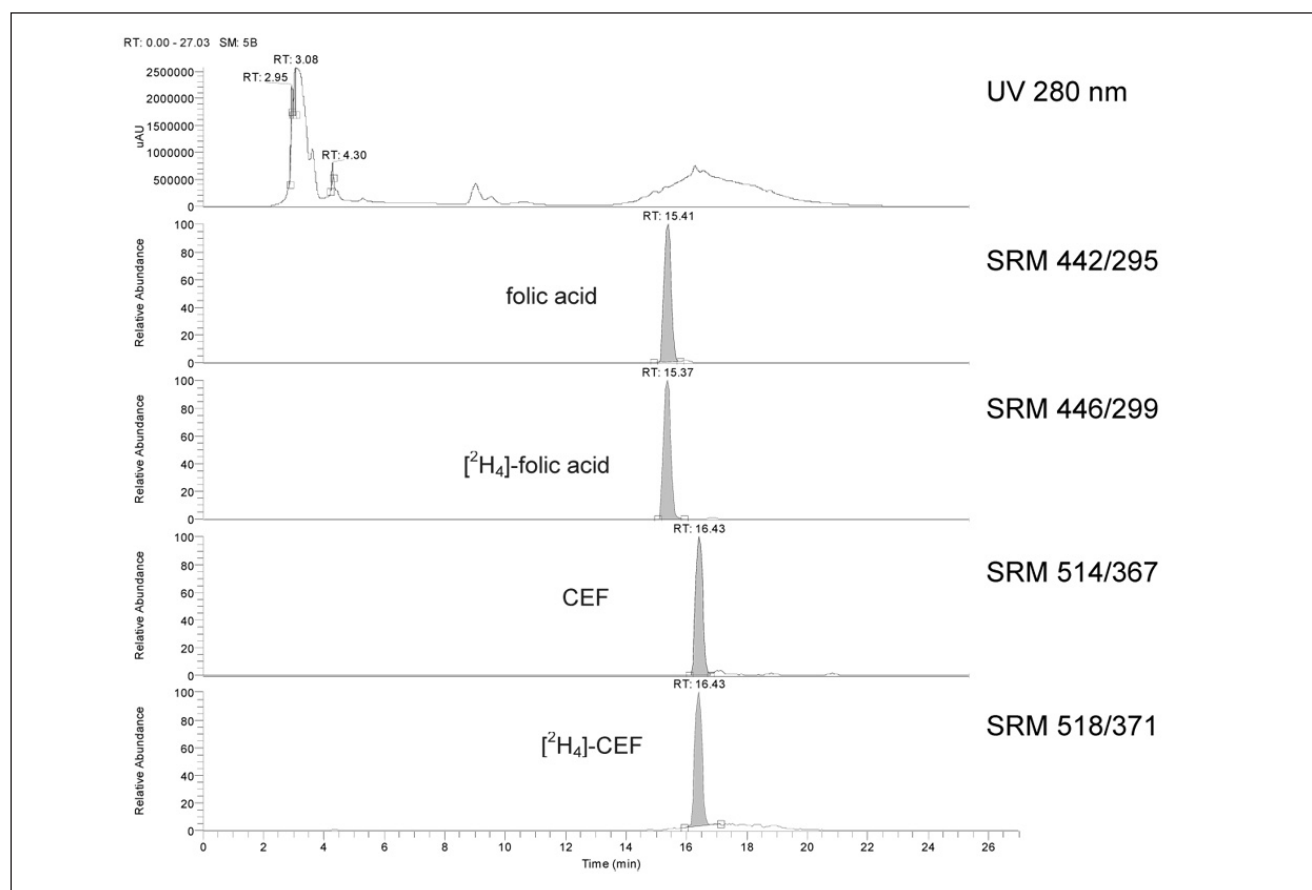


Fig. 1: MS/MS chromatograms of a cookie extract in positive electrospray ionization mode after collision-induced dissociation of the protonated molecules. Unlabeled folic acid (FA), unlabeled N^2 -[1-(1-carboxyethyl)]folic acid (CEF), the internal standards $[^2H_4]$ -FA and $[^2H_4]$ -CEF are detected in the traces SRM 442/259, 514/367, 446/299, and 518/371, respectively. UV: UV absorption selected reaction monitoring (SRM) traces: m/z precursor ion/m/z product ion.

Regarding CEF, we could not detect any traces of CEF (Table 1) in most of the samples under study (Fig. 2). However, cookies purchased in the USA were found to contain considerable amounts of CEF. In particular those made from fortified flour and glucose, glucose syrup or fructose syrup revealed concentrations ranging from 5.1 to 7.1 µg/100 g CEF. In contrast to this, we could not detect CEF in cookies produced either from non-fortified flour or containing artificial sweeteners instead of sugars.

Carboxyethylated products of endogenously occurring folates in foods

Analogously to folic acid, 5-methyltetrahydrofolic acid was reacted with DHA to give N²-[1-(1-carboxy)ethyl]-5-methyltetrahydrofolic acid (CEMTHF). However, in the reaction mixture we could not detect CEMTHF using LC-MS in the full scan and product scan mode (Fig. 3).

To verify or falsify a possible formation of CEMTHF in foods, we analysed strawberry jams, which are high in 5-methyltetrahydrofolic acid and, due to thermal

treatment and sugar addition, could be assumed to contain CEMTHF. Therefore, we applied the highly specific LC-MS/MS method also to these products and adjusted the MS parameters to detect CEF and CEMTHF. However, none of these compounds were detectable. Obviously, 5-methyltetrahydrofolic acid cannot easily be carboxyethylated, which might be due to the lower reactivity of its amino moiety as the aromatic system is less expanded than in FA.

Conclusion

In particular folic acid is a common vitamin for food fortification in order to prevent the aforementioned disorders. Considering the recommended intakes of 400 µg/d as well as 600 µg/d for pregnant and lactating woman and the tolerable upper intake level of 1000 µg/d [15], consumers have to rely on the labelled content to make their diet meet the recommendations. However, our results indicate, that differences for FA up to 110 % above and 20 % below the label claim occur in multivitamin products. Therefore, the manufacturers are called upon adjusting the folate

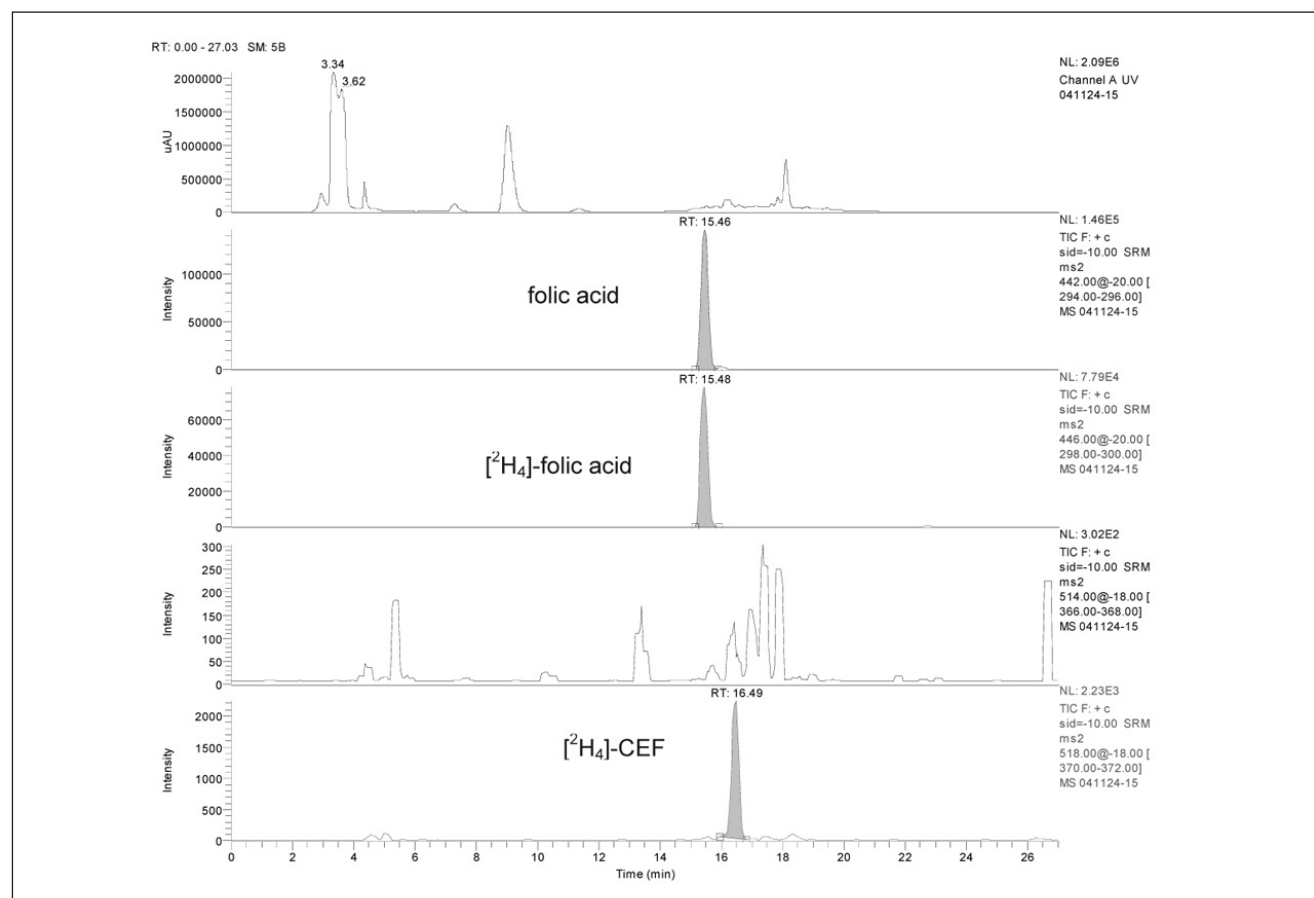


Fig. 2: MS/MS chromatograms of a multivitamin juice extract in positive electrospray ionization mode after collision-induced dissociation (CID) of the protonated molecules. Description see legend of Fig. 1. The signal of unlabelled CEF is not distinguishable from background noise.

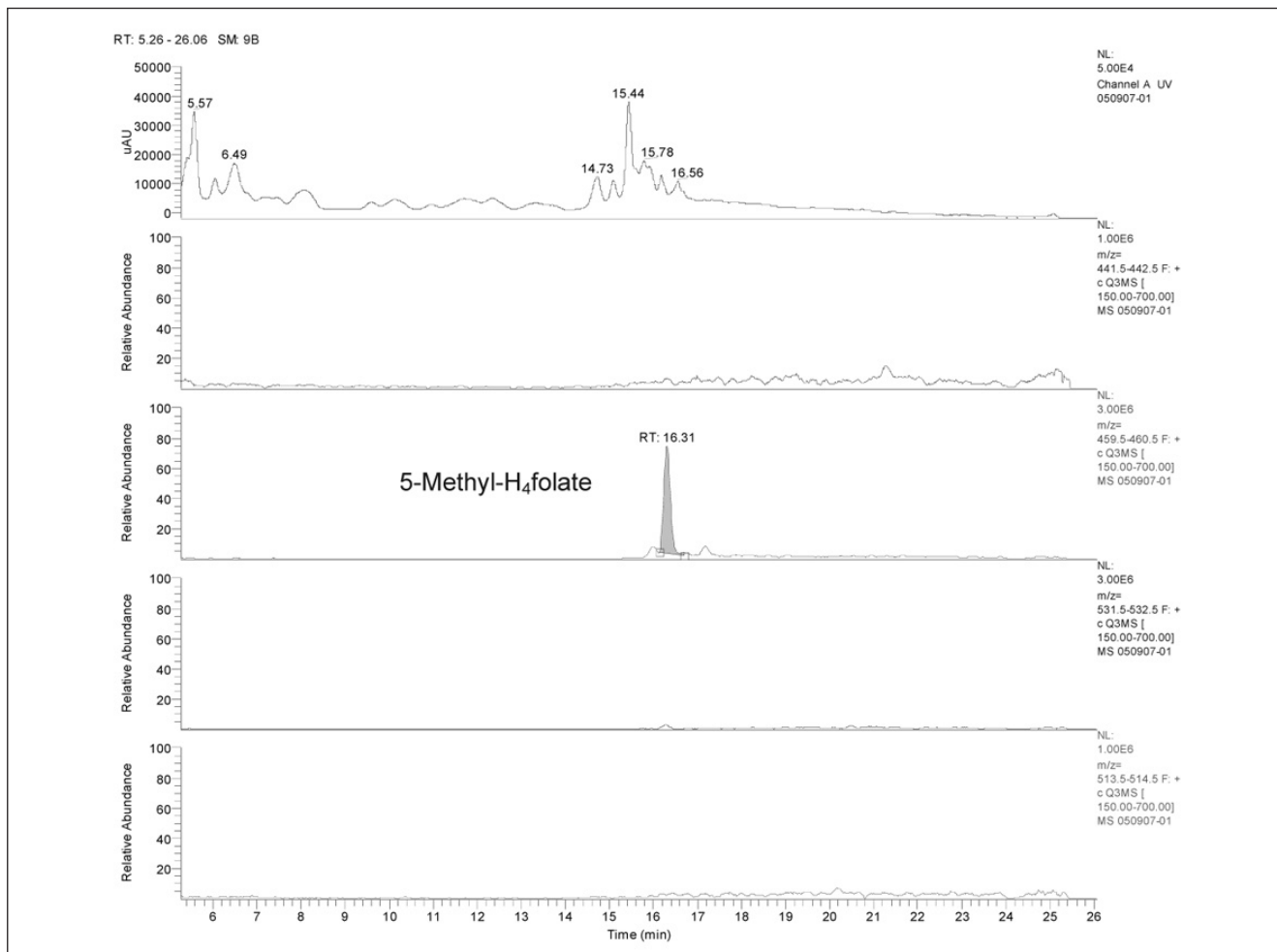


Fig. 3: LC-MS chromatograms of a reaction mixture of 5-methyltetrahydrofolate and dihydroxyacetone. The only folate present is residual 5-methyltetrahydrofolate (5-MeH₄folate) at m/z 460. Neither folic acid (m/z 442), nor carboxyethylfolate (m/z 514), nor carboxyethyl-5-methyltetrahydrofolate (m/z 532) is detectable.

contents more accurately and the official laboratories upon controlling the contents more frequently.

The newly developed SIDA for CEF proved the occurrence of this glycation product of folic acid in commercial cookies. It has to be expected that this reaction contributes to the decrease of folic acid in these products. Therefore, baked products should be made from sucrose rather than from glucose or fructose, when a maximum of folic acid has to be retained. In particular, heated products for diabetics made from fructose or foods made from HFCS may contain significant amounts of CEF. The consequences of this finding are, up to date, an open question and partly depend on the yet unknown physiological properties of CEF.

The possible actions may range from equal vitamin activity as the other folates to inhibition of folate transporters or folate dependent enzymes. Whereas the former would render the formation of CEF uncriti-

cal, the latter would be fatal to folate fortification. For analysis of folates, occurrence of CEF might have significant impacts as well. As the microbiological assay (MA) is a standard method for folate analysis, the response of the employed microorganisms to CEF may be decisive for the total folate content determined by MA. Therefore, further studies of the effects of CEF on the growth of microorganisms, of its bioavailability and toxicity are under way.

In contrast to FA, the endogenously occurring 5-methyltetrahydrofolate does not react to the respective carboxyethyl derivative. However, other maillard reaction products cannot be excluded and the search for these compounds currently is under way.

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