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Quantitation of N^2-[1-(1-Carboxy)ethyl]folic Acid, a Nonenzymatic Glycation Product of Folic Acid, in Fortified Foods and Model Cookies by a Stable Isotope Dilution Assay

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A stable isotope dilution assay (SIDA) for the quantitation of N^2-[1-(carboxy)ethyl]folic acid (CEF) has been developed by using [$^2H_4$]CEF as the internal standard. After sample cleanup by anion exchange chromatography, the three-dimensional specifity of liquid chromatography−tandem mass spectrometry enabled unequivocal determination of the nonenzymatic glycation product of folic acid (FA). When CEF was added to cornstarch, the detection limit for CEF was found to be 0.4 μg/100 g, and a recovery of 98.5% was determined. In analyses of cookies, the intra-assay coefficient of variation was 8.0% ($n = 5$). Application of the SIDA to commercial cookies produced from wheat flour fortified with FA revealed CEF contents of up to 7.1 μg/100 g, which accounted for ~10−20% of the cookies' FA content. In baby foods, multivitamin juices, and multivitamin sweets, however, CEF was not detectable. Further studies on CEF formation during baking of cookies made from fortified flour and different carbohydrates revealed that fructose was most effective in generating CEF followed by glucose, lactose, and sucrose with 12.5, 3.9, 2.5, and 2.5 μg/100 g of dry mass, respectively. During baking, ~50% of FA was retained for both monosaccharides fructose and glucose, and 77% as well as 85% of its initial content was retained for the disaccharides lactose and sucrose, respectively. Of the degraded amount of FA, CEF comprised 28% for fructose as well as 18, 12, and 8% for sucrose, lactose, and glucose, respectively. Therefore, CEF can be considered an important degradation product of FA in baked foods made from fructose. To retain a maximum amount of FA, products should rather be baked with sucrose than with reducing carbohydrates.

KEYWORDS: Fortified foods; folic acid; carboxyethyl folic acid; Maillard reaction; nonenzymatic glycation; stable isotope dilution assay

INTRODUCTION

Due to increasing evidence of their role in physiology, attention in nutrition sciences has been focusing on the vitamins of the folate group. Folate insufficiency appears to contribute to many disorders, for example, megaloblastic anemia in severe cases of deficiency (*1*) as well as the increased incidence of neural tube defects (*2*). Moreover, growing evidence suggests that the incidence of cardiovascular disease (*3*) and Alzheimer's disease (*4*) may be associated with an inadequate folate intake. Furthermore, the role of folate deficiency in inducing single-strand breaks of DNA (*5*) and in favoring the activation of proto-oncogenes (*6*) recently has been 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 (*7*).

This knowledge has spurred mandatory fortification with folic acid (FA) in several countries, especially on the American continent. As it is added to wheat flour, FA becomes a component of pastries as well as other cereal-containing foods and, therefore, often undergoes thermal treatment. Although FA is relatively stable upon heating, its reactions with other ingredients in foods have to be considered.

In this way, recently a Maillard-like reaction involving carbohydrates and FA acting as an amino compound has been reported (*8*). The product, N^2-[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. Reducing sugars such as fructose, glucose, maltose, and lactose produced considerable amounts of CEF; interestingly the highest amounts appeared in models containing the disaccharide maltose or lactose. As sugar products such as corn syrup or high-fructose corn syrup (HFCS) are common sweeteners in baked goods or fruit juices, the question arises about the occurrence of CEF in such foods.

CEF reportedly exhibits spectral properties similar to those of FA (*8*), and, therefore, HPLC using UV detection appears to be a suitable method for its quantitation. However, in extracts

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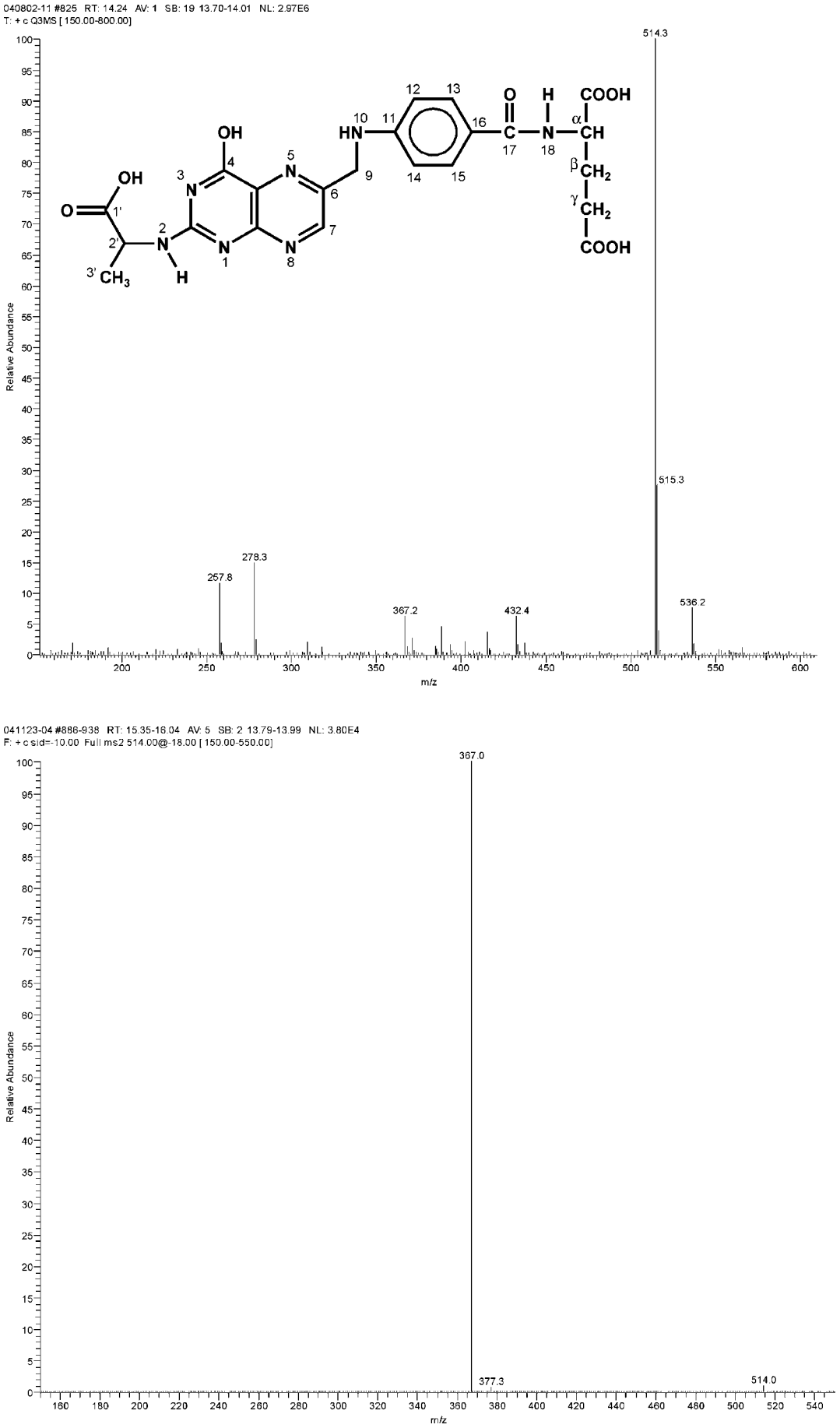

Figure 1. Mass spectrum (top) of N^2-[1-(1-carboxy)ethyl]folic acid (CEF) and MS/MS spectrum (bottom) upon collision-induced dissociation (CID) of the protonated molecule in positive electrospray ionization mode.

of baked products, a significant amount of interferences has to be expected, and a sensitive detection by measuring UV absorption might be difficult.

As we recently reported on the analysis of folates (*9*), the use of stable isotopomers of folate vitamers enables correction for losses during extraction, cleanup, HPLC, and MS detection. The purpose of the present study was, therefore, first to develop a sensitive and accurate method for CEF quantification and, second, to screen commercial products for their content of CEF.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained commercially from the sources given in parentheses: acetonitrile, dihydroxyacetone, folic acid, formic acid, fructose, glucose, 2-mercaptoethanol, lactose, methanol, $NaHCO_3$, KH_2PO_3, Na_2HPO_3, sodium acetate, sodium chloride, and sucrose (Merck, Darmstadt, Germany); CHES, HEPES, and sodium ascorbate (Sigma, Deisenhofen, Germany).

[$^2H_4$]Folic acid was synthesized as reported recently (*10*).

Extraction and Incubation Buffer. For storage and extractions of folates, the following buffer system according to Wilson and Horne (*11*) was used, henceforth referred to as extraction buffer: aqueous HEPES (50 mmol/L) and aqueous CHES (50 mmol/L) at pH 7.85 containing sodium ascorbate (2%) and 2-mercaptoethanol (20 mM).

Syntheses of Unlabeled and Labeled Carboxyethyl Folic Acid. CEF was prepared according to the procedure described by Schneider et al. (*8*) with a slightly different cleanup of the raw synthesis.

FA (0.5 mmol) was reacted with dihydroxyacetone (2 mmol) in phosphate buffer (1 mol/L, pH 7.4) at 100 °C for 24 h. Then, aliquots (10 μL) of the mixture were subjected to preparative HPLC using a Hyperclone ODS column (5 μm, 250 × 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. CEF was eluted as the highest peak at 280 nm and was collected from 50 runs. The pooled fraction finally was lyophilized.

The purified compound showed NMR spectra in [$^2H_6$]DMSO very similar to those reported by Schneider et al. (*8*).

^{1}H NMR ([$^2H_6$]DMSO) δ 1.39 [3 H, d, H−C(3′), $^3J_{3'\,2'} = 7.0$ Hz], 1.88−2.06 (2 H, m, H−C(β)], 2.30 [2 H, t, H−C(γ)], 4.29−4.36 [m, 1 H, H−C(α), 1 H, H−C(2′)], 4.49 [2 H, d, H−C(9), $^3J_{9\ 10} = 5.9$ Hz)], 6.64 [2 H, d, H−C(12), H−C(14), $^3J_{12/14\ 13/15} = 8.7$ Hz], 6.90 [1 H, t, H−N(10); $^3J_{10\ 9} = 6.0$ Hz], 7.61 [2 H, d, H−C(13), H−C(15), $^3J_{13/15\ 12/14} = 8.6$ Hz], 8.03 [1 H, d, H−N(18), $^3J = 7.4$ Hz], 8.64 [1 H, s, H−C(7)].

^{13}C NMR ([$^2H_6$]-DMSO) δ 18.56 [C(3′)], 27.08 [C(β)], 31.23 [C(γ)], 45.93 [C(9)], 50.55 [C(2′)], 52.45 [C(α)], 111.23 [C(12), C(14)], 121.69 [C(16)], 128.14 [C(4a)], 128.76 [C(13), C(15)], 148.31 [C(7)], 150.66 [C(11)], 151.65 [C(8a)], 151.83 [C(2)], 156.26 [C(6)], 161.02 [C(4)], 164.73 [C(17)], 174.51 [C(δ)], 174.82 [C(1′)].

MS and MS-MS spectra of the protonated molecule in positive electrospray ionization mode are shown in **Figure 1**.

The molar extinction coefficients at pH 7.0 in phosphate buffer were 18.8×10^3 and 6.2×10^3 L/(mol cm) at 284 and 360 nm, respectively.

[$^2H_4$]-N^2-[1-(1-Carboxy)ethyl]folic acid acid was synthesized analoguously to the unlabeled compound by starting with [$^2H_4$]folic acid.

Commercial Food Samples. Two multivitamin juices and four baby foods were obtained from local retail stores in the city of Munich, Germany. The baby foods were infant formulas from different producers. Six different types of commercial cookies were purchased at supermarkets in Washington, DC.

Baking of Model Cookies. Cookies were produced by mixing wheat flour (25 g) fortified with FA (140 μg/100 g) with a leavening agent (1 g) consisting of KH_2PO_3 and sodium hydrogencarbonate, baking margarine (25 g), water (8 g), and one of the carbohydrates glucose, fructose, sucrose, or lactose (25 g), respectively. After refrigeration for 60 min at 6 °C, the dough was rolled to a thickness of 2 mm, and round cookies were cut out with a diameter of 45 mm. The raw cookies were baked in an oven at 175 °C for 5, 10, and 15 min, respectively. Subsequently, the cookies were allowed to cool and stored at −60 °C until analysis.

Table 1. Collision-Induced Dissociation Conditions for Detection of Folic Acid and N^2-[1-(1-Carboxy)ethyl]folic Acid (CEF)

compound	precursor ion	product ion	collision energy (%)
folic acid	442	295	20
[$^2H_4$]folic acid	446	299	20
CEF	514	367	18
[$^2H_4$]CEF	518	371	18

Extraction and Quantification of CEF in Foods. Solid samples were frozen in liquid nitrogen and minced in a blendor (Privileg, Quelle, Fürth, Germany). The resulting powders or liquid samples (1 g) were mixed with extraction buffer (10 mL) containing [$^2H_4$]FA (1 μg) and [$^2H_4$]CEF (0.1 μg). Then, the mixtures were stirred for 1 h and subsequently centrifuged for 10 min at 13000 rpm. The supernatant was then passed through a syringe filter (0.4 μm, Millipore, Bedford, MA) or subjected to SPE prior to LC-MS/MS.

Sample Cleanup by Solid-Phase Extraction (SPE). Extracts were purified by SPE according to the method described by Gounelle et al. (*12*), using a 12-port vacuum manifold (Alltech, Bad Segeberg, Germany) equipped with Bakerbond SAX cartridges (quaternary amine, 500 mg, no. 7091-3, Baker, Gross-Gerau, Germany). The cartridges were successively activated with 2 volumes of hexane, methanol, and water and then conditioned with 7−8 volumes of phosphate buffer (pH 7.5, 0.01 mol/L, containing 0.2% 2-mercaptoethanol).

After application of the sample extracts (6 mL), the columns were washed with 6 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). One hundred microliters of 2-mercaptoethanol 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 × 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).

The mobile phase consisted of variable mixtures of aqueous formic acid (0.1%; eluent A) and acetonitrile (eluent B) at a flow of 0.4 mL/min. Gradient elution started at 10% B for 6 min, followed by raising the concentration of B linearly to 100% within 19 min. Subsequently, the mobile phase was maintained at 100% B over 5 min before the column was equilibrated for 5 min at the initial mixture.

During the first 10 min of the gradient program, the column effluent was diverted to waste. The spectrometer was operated in the positive electrospray mode using selected-reaction monitoring (SRM). The spray voltage was set to 5.5 kV, the capillary temperature to 200 °C, and the capillary voltage to 24.3 V. Collision-induced dissociation (CID) was performed using a source CID collision energy of 10% and the further conditions detailed in **Table 1**.

Determination of Response Factors for LC-MS/MS. Solutions of CEF and [$^2H_4$]CEF in extraction buffer (10 mL) were mixed in five mass ratios ranging from 0.2 to 5 to give a total CEF content of 2 μg. Subsequently, the CEF mixtures were subjected to LC-MS/MS as outlined before. Response factors, R_f, were calculated as reported recently (*13*) and gave $R_f = 0.74$ in the SRM transitions 514/367 and 518/371.

Determination of Detection Limits (DL) and Quantification Limits (QL). Amounts of 10, 20, 40, and 80 ng of CEF were added to edible cornstarch (1 g) and analyzed as detailed before in triplicates. DL and QL were calculated according to the method of Hädrich and Vogelgesang (*14*): DL is the concentration calculated from the maximum height of the 95% confidence interval at the zero addition level; QL is the addition level for which the lower 95% confidence limit equals the upper 95% confidence limit of the addition level at the DL.

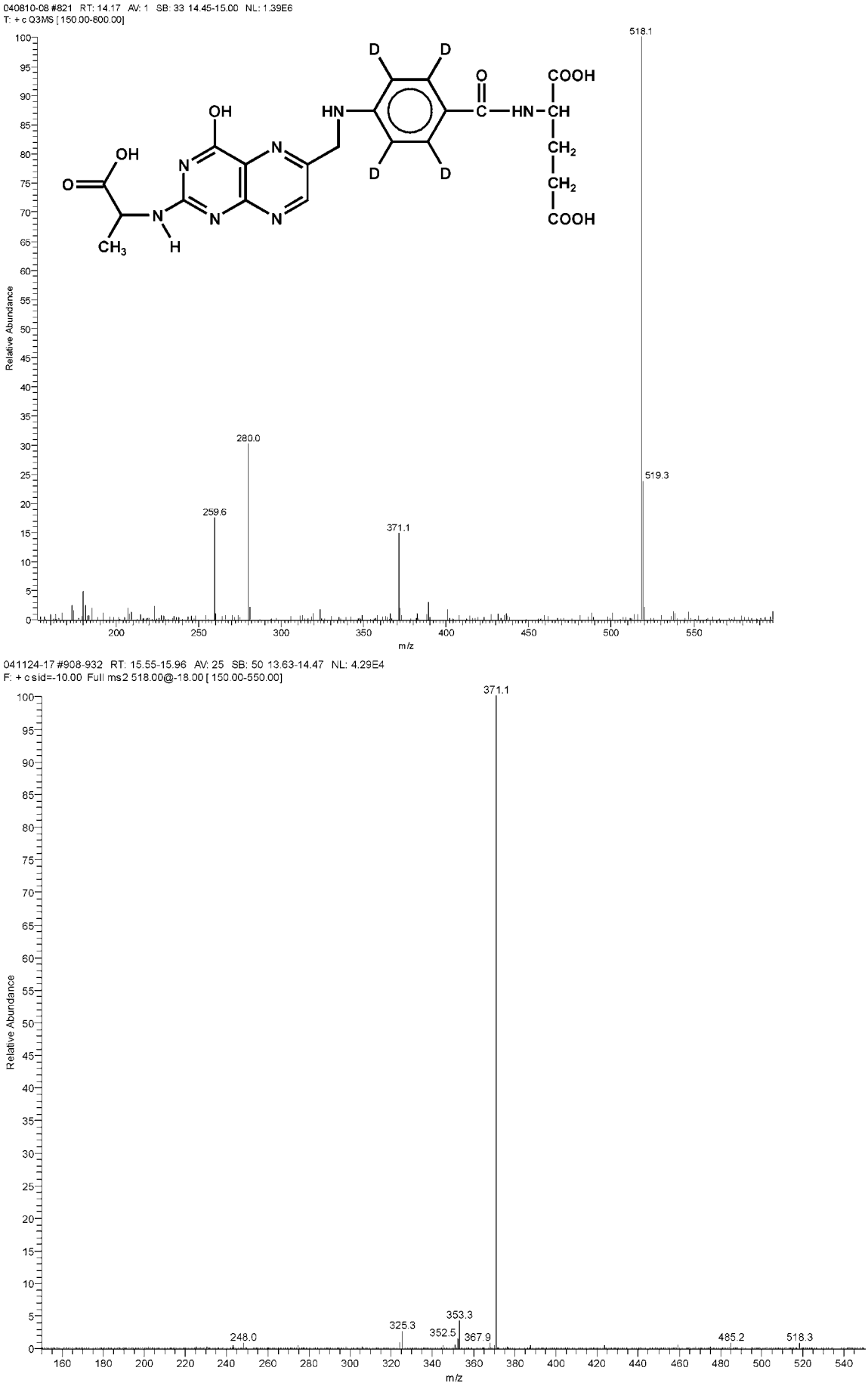

Figure 2. Mass spectrum (top) of [$^2H_4$]-*N*^{2}-[1-(1-carboxy)ethyl]folic acid ([$^2H_4$]CEF) and MS/MS spectrum (bottom) upon CID of the protonated molecule in positive electrospray ionization mode.

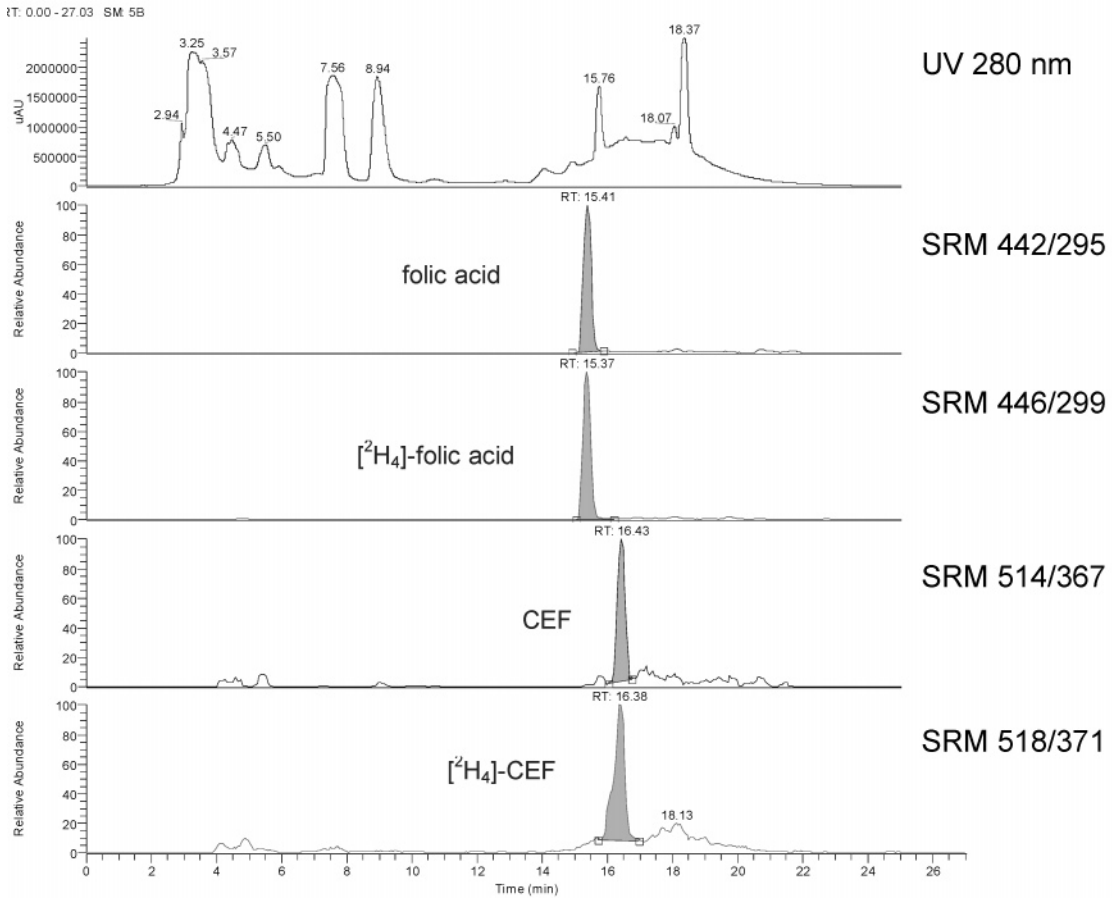

Figure 3. MS/MS chromatograms of a cookie extract without cleanup by anion exchange chromatography in positive electrospray ionization mode after CID of the protonated molecules. Unlabeled folic acid (FA), unlabeled CEF, and the internal standards [$^2H_4$]FA and [$^2H_4$]CEF are detected in the traces SRM 442/295, 514/367, 446/299, and 518/371, respectively. UV, UV absorption. Selected reaction monitoring (SRM) traces: *m*/*z* precursor ion/*m*/*z* product ion.

Precision and Recovery. Intra-assay precision was evaluated by analyzing cookies as detailed before. Recovery was determined by adding 160 ng of CEF to edible cornstarch (1 g) and performing a stable isotope dilution assay (SIDA) as detailed before in quadruplicate analysis.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR spectra and COSY were recorded with an AMX-400 III (Bruker, Karlsruhe, Germany) at a frequency of 400.13 MHz. ^{13}C NMR spectra and HMQC were measured with an AMX-500 (Bruker) at a frequency of 500.13 MHz. Shifts are expressed in parts per million downfield from tetramethylsilane in [$^2H_6$]DMSO.

Determination of Dry Matter of Cookies. Dry matter of the model cookies was determined according to method L 02.06 of the official collection of test methods according to article 35 of the German food law (*15*) using the cookie powders prepared for SIDAs.

Color Measurement. The CIE-$L^*a^*b^*$ coordinates of the cookies' surfaces were measured with a spectrophotocolorimeter spectrocolor pen (Dr. Lange, Berlin, Germany) and calculated by using the software spectral-QV 3.5. The spectrocolor pen was calibrated with a white tile at 20 °C ($L^* = 95.3$, $a^* = -0.6$, $b^* = 1.4$).

RESULTS AND DISCUSSION

During baking of doughs prepared from flour fortified with folic acid, losses of FA have been reported (*16*).

Besides cleavage of the bond between C-9 and N-10, which produces 4-aminobenzoylglutamic acid, losses by reaction with carbohydrates have been shown in model systems (*8*). In the latter study, CEF has been identified as a major degradation product.

Synthesis of CEF. As CEF is not commercially available, initial experiments were run to synthesize it as a reference compound. To avoid the tedious HPLC purification reported by Schneider et al. (*8*), we first investigated alternative ways. A conceivable path to CEF was the procedure for N^6-[1-(1-carboxy)ethyl]lysine reported by Krook et al. (*17*) by reacting FA with sodium pyruvate and subsequent reduction with $NaBH_4$. Alas, this procedure did not give yields higher than 5%. Therefore, we followed the way described by Schneider et al. by reacting FA with dihydroxyacetone (DHA) and purifying CEF from unreacted FA by preparative HPLC. NMR experiments revealed similar data as reported by the latter authors. In particular, the ^{1}H NMR doublet signal of the methyl moiety at 1.39 ppm coupling to the methine group at 4.3 ppm confirmed the presence of the carboxyethyl group.

For quantitation of diluted solutions of pure CEF we used its molar extinction coefficient ϵ at pH 7 and 284 nm of 18.8×10^3 L/(mol cm).

LC-MS Properties of CEF. Our earlier studies have shown that LC-MS/MS detection in combination with a SIDA is a sensitive and accurate tool for the quantitation of folates (*6*). In acidic eluents, folates form protonated molecules and dissociate upon MS/MS by loss of glutamic acid. Expectedly, CEF behaved similarly upon CID (**Figure 1**). However, the intensity in LC-MS and LC-MS/MS was 1 order of magnitude lower than that of FA, thus indicating that carboxyethylation of the exocyclic amino moiety lowers decisively the ionization yield. This effect might be due to a decreased pK_a resulting from the

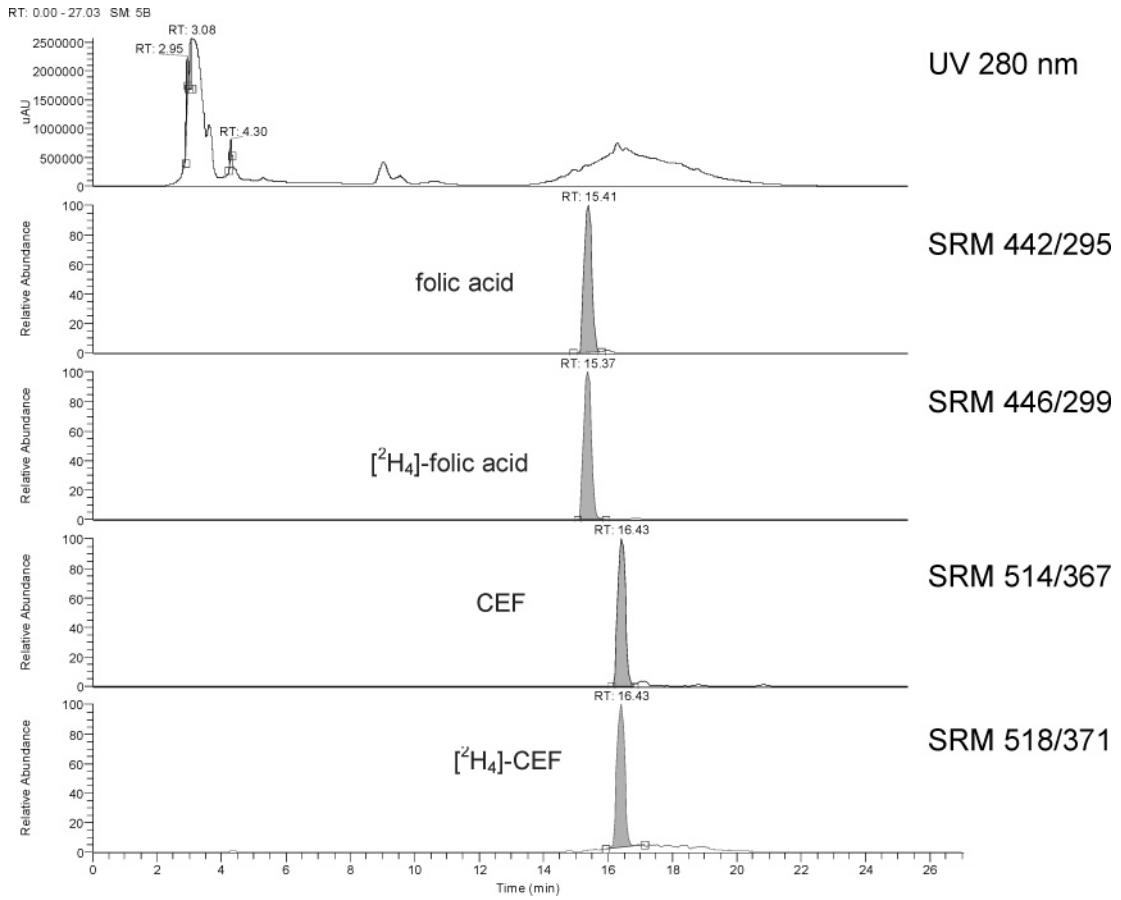

Figure 4. MS/MS chromatograms of a cookie extract with cleanup by anion exchange chromatography in positive electrospray ionization mode after CID of the protonated molecules. For a description, see the caption of **Figure 3**.

additional acidic moiety in the molecule or from the lower basicity of N-2 due to the electronegative effect of the carboxyethyl group. Therefore, it can be assumed that ionization of underivatized folates occurs widely via protonation at N-2.

Synthesis of Labeled CEF. During the development of a SIDA for folates, we had been starting with the synthesis of deuterated FA to generate the $^2H_4$-labeled isotopomers of the most important folate vitamers that occur endogenously in foods (*10*). Therefore, it appeared to be straightforward to synthesize labeled CEF by starting from [$^2H_4$]FA, too. Synthesis of [$^2H_4$]-CEF was performed analoguously to that of unlabeled CEF by reacting [$^2H_4$]FA with DHA and subsequent purification by preparative HPLC. LC-MS and LC-MS/MS analyses confirmed the mass increment of 4 Da in both the protonated molecule and the product ion after loss of glutamic acid upon CID as shown in **Figure 2**.

Development of a SIDA. To convert the measured ion intensities into the mass ratios of labeled and unlabeled 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.

As the CEF content in foods was expected to be quite low, the sensitivity of LC-MS was evaluated by determining the DL in edible starch according to the method of Hädrich and Vogelgesang (*14*). The calculations resulted in a DL of 0.4 μg/100 g and a QL of 1.3 μg/100 g in cereal-based foods. Recovery was evaluated by adding 16 μg/ 100 g to edible starch and was found to be 98.5%. Intra-assay precision was determined by analyzing CEF in cookies and revealed a CV of 8.0% ($n = 5$).

To avoid interferences in LC-MS/MS detection, we used anion exchange chromatography (AEC) for purification of the extracts. From **Figures 3** and **4** it can be seen that AEC provided extracts with less background noise than those obtained without sample cleanup. Interestingly, the intensity ratio between FA and CEF after AEC was very similar to that without using this cleanup, thus indicating that CEF was not discriminated when binding to the anion exchange material.

To exclude a deuterium−protium exchange during extraction and cleanup, we spiked a cookie sample devoid of folates with CEF and analyzed it in duplicate. The intensity ratios of the isotopomers recorded (a) before and (b) after extraction and sample cleanup were not distinguishable ($P < 0.001$), thus indicating that no protium−deuterium exchange had occurred.

The formation of CEF in model systems renders it a conceivable component in foods that are similarly composed and undergo a similar treatment like the models used by Schneider et al. (*8*). Possible examples are multivitamin juices containing carbohydrates and pasteurized to become shelf-stable. Furthermore, cookies produced from folate-fortified flour and glucose or fructose syrup may contain CEF. Therefore, we applied the newly developed SIDA to these kinds of products. In the first group consisting of two multivitamin juices, we could not detect any traces of CEF (**Figure 5**; **Table 2**). The same result was obtained for multivitamin sweets and fortified milk products such as baby foods. However, cookies purchased in the United States 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 of CEF/100 g. In contrast to this,

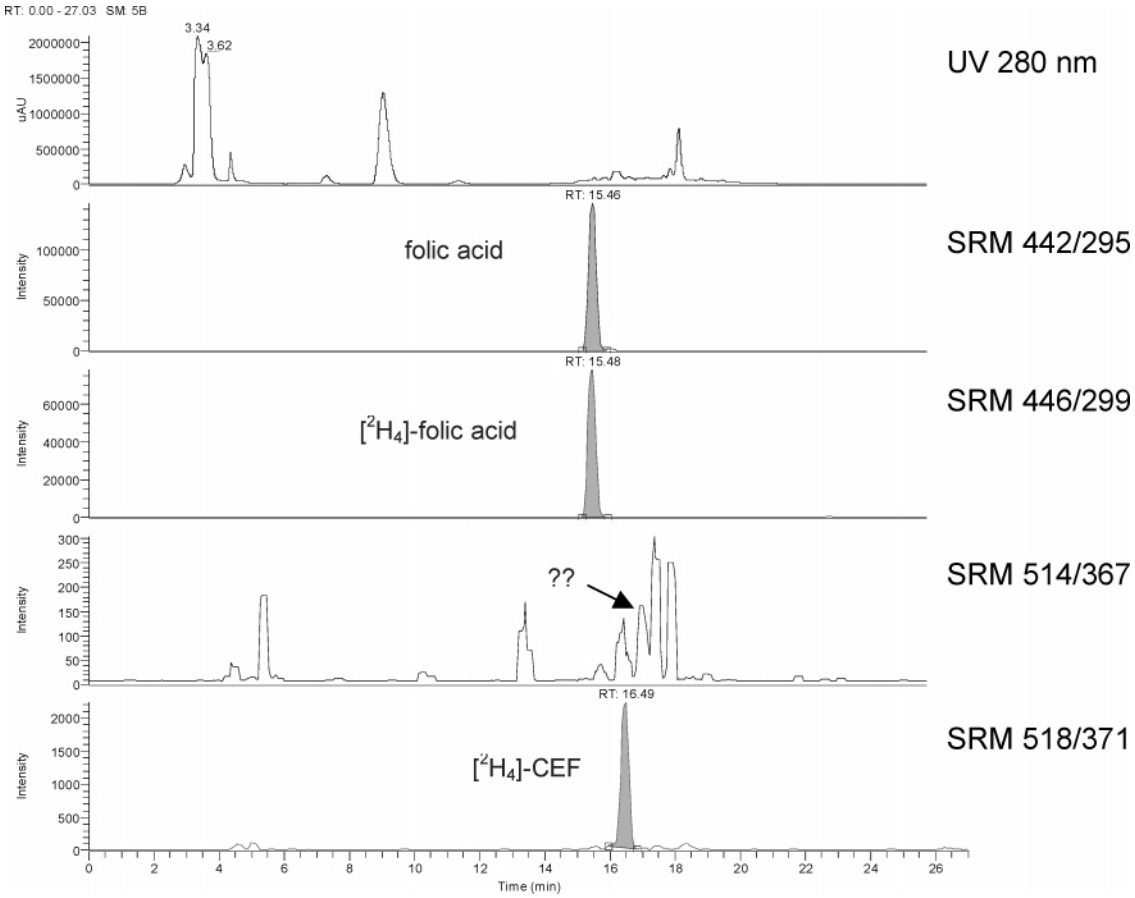

Figure 5. MS/MS chromatograms of a multivitamin juice extract in positive electrospray ionization mode after CID of the protonated molecules. For a description, see the caption of **Figure 3**.

Table 2. Contents of Folic Acid and N^2-[1-(1-Carboxy)ethyl]folic Acid (CEF) in Different Foods Fortified with Folic Acid

sample	no. of samples	folic acid (μg/100 g)	CEF (μg/100 g)
multivitamin juices	2	51–77	nd[a]
baby food	4	48–137	nd
multivitamin sweets	2	670–700	nd
cookies	3	32–103	5.1–7.1
cookies, sugar-free	2	55–68	nd
cookies made from non-fortified flour	1	0.4	nd

[a] Not detectable.

we could not detect CEF in cookies produced either from nonfortified flour or containing artificial sweeteners instead of sugars.

To get a more detailed insight into the conditions that favor CEF formation in cookies, we baked model cookies by using flour fortified with FA and different carbohydrates. As Schneider et al. (*8*) reported that in model solutions reducing disaccharides such as lactose or maltose most effectively produced CEF, we baked cookies that contained lactose besides the common carbohydrates glucose, fructose, and sucrose. As expected, CEF formation was more pronounced in the cookies baked for the longest time of 15 min compared to those baked for 10 or 5 min and to the raw dough (**Figure 6**). However, in contrast to the results reported by Schneider et al. (8), fructose was the most effective CEF precursor followed by glucose, lactose, and sucrose. Regarding the retained content of FA during baking listed in **Table 3**, the reducing monosaccharides glucose and fructose were most effective in depleting FA by ~50% of its initial content, whereas the disaccharide lactose reduced FA only by 23% and the nonreducing sucrose showed even lower reduction of FA. Thus, the principal effectiveness of reducing sugars and ineffectiveness of nonreducing sugars, such as sucrose, in the Maillard reaction were confirmed. The observation that in sucrose cookies CEF was formed and FA was degraded after 15 min of baking might be attributed to cleavage of sucrose to the reactive sugars fructose and glucose. Comparing the loss of FA with the formation of CEF, for fructose CEF

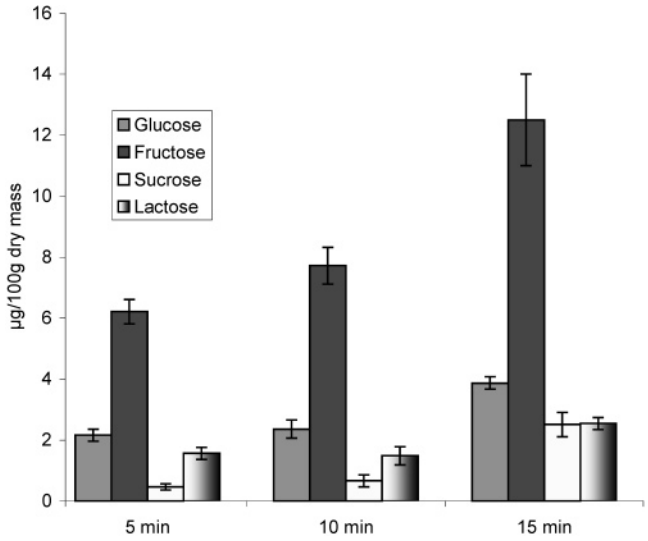

Figure 6. Contents of CEF in model cookies made from different carbohydrates and from flour fortified with FA after various baking times. Stable isotope dilution assays were performed in triplicates.

Table 3. Retained Amounts, Losses of Folic Acid (FA), and Relative Percentages Thereof Attributable to Formation of N^2-[1-(1-Carboxy)ethyl]folic Acid (CEF) in Model Cookies after 5, 10, or 15 min of Baking Time Determined by Stable Isotope Dilution Assays ($n = 3$)[a]

	glucose			fructose			sucrose			lactose		
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
absolute and relative[b] retained contents of FA (μg/100 g of dry matter)	76 (85.2)	75.4 (84.5)	39.1 (43.9)	73.6 (82.4)	63.1 (70.7)	45.3 (50.7)	91.0 (100)	89.4 (100)	75.7 (84.8)	81.9 (91.8)	82.1 (92.0)	68.7 (77.0)
absolute loss of FA (μg/100 g of dry matter)	13.2	13.8	50.1	15.7	26.1	44.0	0	0	13.6	7.3	7.1	20.5
percentage attributable to CEF formation	16	17	8	40	30	28	0	0	18	21	21	12
L^*	77.3	61.4	44.9	73.5	61.2	46.0	77.9	70.9	60.4	83.1	72.7	60.6
a^*	1.8	11.4	13.3	3.9	11.9	15.4	0.85	5.60	11.4	0.89	7.14	10.2
b^*	20.4	32.3	25.4	24.7	33.6	30.1	17.2	27.0	29.1	13.4	29.4	29.4

[a] Upper surface color of the cookies is given in terms of lightness L^*, a^* value, and b^* value according to the CIE-$L^*a^*b^*$ color system. [b] In relation to an FA content of 89.2 μg/100 g of dry matter in the dough (percentages in parentheses).

contributed to 28% of the total degradation in contrast to percentages lower than 18% for the other carbohydrates under study. It might be speculated that the remaining percentage of loss is due to oxidative processes such as cleaving the bond between N-9 and C-10 to give 4-aminobenzoylglutamate (*18*). It is notable that the degradation of FA due to CEF formation may be limited by the concurring reaction of DHA with amino groups of proteins and of further amino compounds present in the dough. However, the reactivity of DHA toward different amino compounds is yet unknown.

The course and extent of nonenzymatic browning was also followed by the color of the cookies' surfaces. On the basis of the CIE-$L^*a^*b^*$ color system, the lightness L^* of the cookies was most effectively reduced by fructose and glucose followed by sucrose and lactose, which were both similarly light-colored. Obviously, browning of the model cookies went qualitatively in parallel with degradation of FA.

Of all carbohydrates, the paramount impact of fructose in generating CEF compared to the other reducing sugars was interesting. This finding is consistent with the hypothetical pathway of CEF formation from the retroaldol product DHA proposed by Schneider et al. (*8*). As 2-ketoses already bear a 1,3-dihydroxy-2-oxo moiety, DHA preferentially should be formed from the naturally most abundant 2-ketose, namely, fructose. However, to the best of our knowledge, DHA has not yet been quantified in Maillard systems. Therefore, further pathways such as the retroaldol cleavage of primary Maillard products cannot be excluded.

Conclusions. The results of our quantitations revealed that the reaction product of FA with carbohydrates, CEF, is formed during baking of cookies. This reaction obviously contributes to the decrease of FA in these products. Therefore, baked products should be made from sucrose rather than from glucose or fructose when a maximum of FA 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, 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 uncritical, 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. These aspects point to the need for further studies of the effects of CEF on the growth of microorganisms and of its bioavailability and toxicity.

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