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Review

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Occurrence, Stability, and Determination of Formyl Folates in Foods

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ABSTRACT: The B-vitamin folate has specific tasks as a one-carbon (C1) group supplier in the building and repair of DNA and RNA as well as in the methylation of homocysteine to methionine. Folate occurs in all living cells as a dynamic pool of several interconvertible forms carrying different C1 groups. Along the food chain, this dynamic pool of folates constantly changes due to either enzymatic or chemical interconversions during food processing and storage. These interconversions make it difficult to determine individual folate forms in foods. The formyl folates, the second most predominant forms of food folates, after 5-methyltetrahydrofolate, are particularly prone to interconvert at low pH. Today, this knowledge is often neglected, leading to risks for analytical underestimation of formyl folates. The purpose of the review is to explore the stability and interconversions of formyl folates in foods as well as to analyze the pitfalls in the determination of formyl folates.

KEYWORDS: *formyl folates, stability, interconversions, analysis, HPLC, LC-MS*

INTRODUCTION

Folates belong to a group of B vitamins, metabolically active cofactors in the transfer of one-carbon (C1) groups. The vitamin is crucial for all cell divisions and growth by participating in the biosynthesis of RNA and DNA. Pregnancies are associated with increased demands of folate, and suboptimal intakes might cause malformations of the fetus such as neural tube defects. Other health aspects related to low folate status are increased homocysteine levels, a risk factor for cardiovascular diseases, and impaired cognitive functions.[1−5] Furthermore, there seems to be a U-formed risk for cancer − higher risks with low folate intakes[6,7] or with high intakes of folic acid supplements, ≥1 mg/day.[8,9]

Native, biologically active folates occur as reduced tetrahydropteroylpolyglutamates ($H_4PteGlu_n$) substituted with either methyl (CH_3), formyl (HCO), methylene (CH_2), methenyl (CH^+), or formimino (CH=NH) groups, linked to a pteroyl moiety, which in turn is bound to one or several glutamates. Folic acid is the synthetic unreduced and monoglutamic form used as food fortificant (see Figure 1). This form is the most stable one, whereas natural folate forms differ markedly in stability. The most common natural folate in food is 5-methyltetrahydrofolate (5-CH_3-H_4folate), followed by formyl folates, which are the second largest group of food folates. The latter include 5-formyltetrahydrofolate (5-HCO-H_4folate) and 10-formyltetrahydrofolate (10-HCO-H_4folate) as well as their interconversion and oxidation products such as 5,10-methenyltetrahydrofolate (5,10-CH^+=H_4folate), 10-formyl-dihydrofolate (10-HCO-H_2folate), and 10-HCO-folic acid. The stability of formyl folates strongly depends on pH, oxidation, and heating. Tetrahydrofolate (H_4folate) is one of the least stable folate forms and occurs in foods in minor amounts.

The microbiological method implemented during the 1960s is still today the only certified analytical method for food samples containing different folate forms.[10] The microbiological assay responds slightly differently to all bioactive folate forms. Despite its high sensitivity, the microbiological method has some serious drawbacks; it has a narrow calibration range, and the analytical procedure is tedious and time-consuming and is not able to distinguish between different folate forms, which make some folate data in food databases uncertain. Consequently, there is a need for more accurate and reliable analytical methods for determining dietary folates.[11,12] With the introduction of liquid chromatography (LC), individual folate forms could be separated and quantified with better accuracy. The most selective and sensitive detector used with LC is mass spectrometric detector. Common mobile phases used in combination with organic solvents are phosphoric acid, formic acid, or acetic acid having pH between 2.3 and 3.4. Furthermore, the cleanup procedure performed prior to LC analysis uses similar buffers. Some of the food folates, in particular the formyl folates, are prone to interconversions into 5,10-CH^+=H_4folates at acidic pH values. Moreover, at neutral and alkaline pH, 5-HCO-H_4folate is stable, whereas 10-HCO-H_4folate exhibits high instability. This latter form is sensitive for oxidation and isomerization at all pH values. 5,10-CH^+=H_4folate is stable only at pH below 2.[11−15] As a consequence, interconversions of formyl and methenyl folates are expected to take place during HPLC and LC-MS analyses. The extent to which these phenomena affect the analytical results and their agreement with true folate content in original food samples is presently unknown.

Because the analysis of the major food folate, 5-CH_3-H_4folate, is easier compared to other more unstable folate forms, we focus this review on the second food folate group, the formyl folates. The aim of this review is to summarize the literature on the occurrence, stability, and interconversions of formyl folates. We will highlight the most critical steps during the analytical procedure and discuss pitfalls in the analysis of formyl folates in foods. We also summarize the role of formyl folates in cell metabolism, which includes a number of enzymatic

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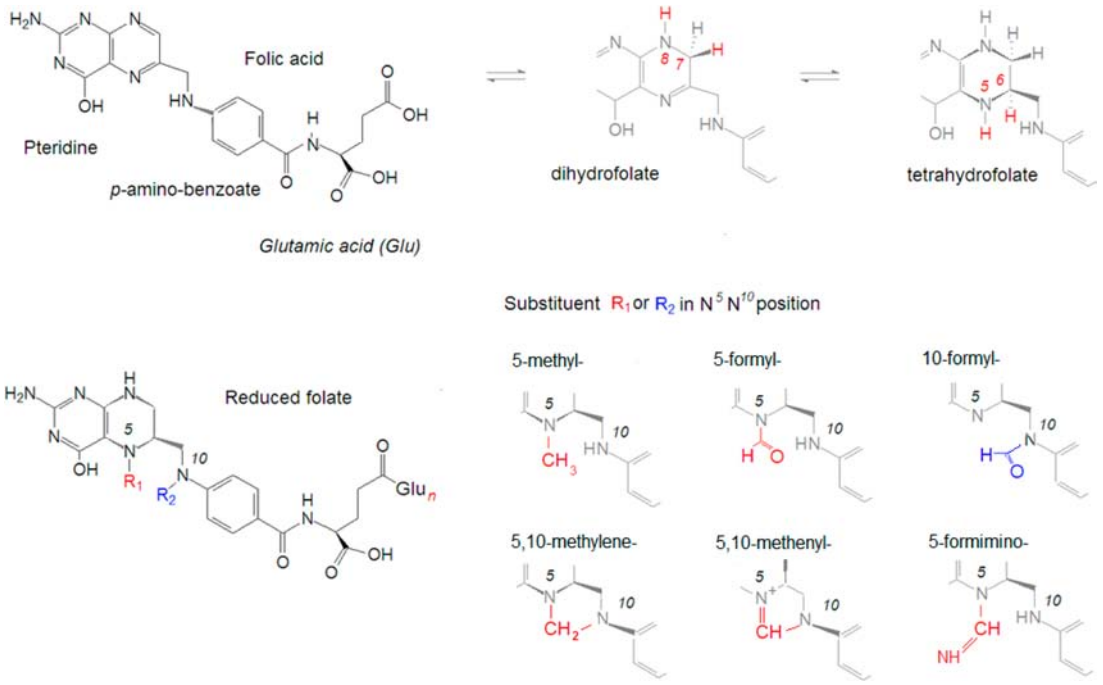

Figure 1. Structures of folic acid and the native folate forms.

interconversions. Analysts need to consider both chemically and enzymatically induced interconversions of formyl folates to correctly interpret their analytical data. With more understanding of stability aspects of formyl folates, the quality of their quantification can be substantially improved.

■ ROLE IN CELL METABOLISM

In all living cells, folates occur mainly as substituted polyglutamic (conjugated) tetrahydrofolates ($H_4PteGlu_n$). Their function is to bind (accept), transport, and donate C1 groups. These C1 groups originate from the catabolism of serine, glycine, histidine, or purines. The accepted C1 groups are then transported and enzymatically interconverted into five different C1 species as illustrated in the simplified scheme of folate metabolism (see Figure 2). Only three of these folate forms participate as direct C1 donors, where two of them, 10-HCO-H_4folate and 5,10-CH_2-H_4folate, have key roles in the repair and novel biosynthesis of DNA and RNA. 10-HCO-H_4folate donates carbon atoms for two sites of the skeleton of each purine molecule, forming two of the four base pairs of the nucleotides in DNA and RNA. 10-HCO-H_4folate also supplies carbon-2 of the imidazole ring in the biosynthesis of histidine.[1,3,16]

5,10-CH_2-H_4folate is involved in another key step in the nucleotide biosynthesis by donating its methylene group to deoxyuridylate (dUMP) to form thymidylate (dTMP), one of the pyrimidines of DNA. DNA and RNA are built up of four base pairs, two purines, adenine and guanine, and two pyrimidines. In RNA the two pyrimidines are uracil and cytosine, whereas in DNA they are thymine and cytosine due to methylation of uracil into thymine. Low cellular folate levels retard methylation of uracil to thymine, leading to thymine depletion and increased uracil concentrations. Because uracil and thymine differ by only a single methyl group, uracil could be included in DNA in place of thymine, which induces DNA strand breaks and chromosomal and genomic instability. These phenomena are believed to explain why low folate intakes are associated with increased cancer risks.[17] Disturbances in DNA and RNA

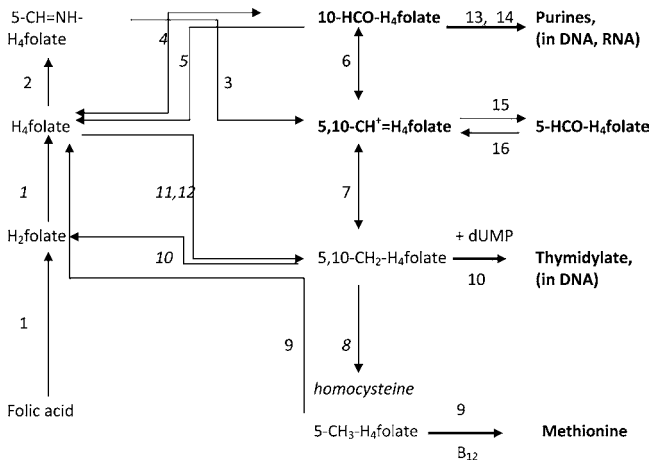

Figure 2. Overview of biochemical pathways of folates.[1,3,4,16,44] (1) Dihydrofolate reductase; (2) glutamate formiminotransferase; (3) formiminotetrahydrofolate cyclodeaminase; (4) 10-formyltetrahydrofolate synthase; (5) 10-formyltetrahydrofolate dehydrogenase; (6) 5,10-methenyltetrahydrofolate cyclohydrolase; (7) 5,10-methylenetetrahydrofolate dehydrogenase; (8) 5,10-methylenetetrahydrofolate reductase; (9) methionine synthase; (10) thymidylate synthase; (11) serinehydroxymethyltransferase 1; (12) serinehydroxymethyltransferase 2; (13) phosphoribosylglycinamide transformylase; (14) aminocarboxamide ribonucleotide transformylase; (15) 5-formyltetrahydrofolate cyclodehydrase; (16) 5,10-methenyltetrahydrofolate synthetase.

biosynthesis retard cell division and normal cell growth, leading to the classical folate deficiency disease, megaloblastic anemia. Low folate intakes during pregnancy are associated with increased risks for neural tube defects and spontaneous abortions.[18]

5,10-CH_2-H_4folate also participates in the biosynthesis of methionine from homocysteine. The remethylation of homocysteine to methionine is an important demethylating route of the folate metabolites and restores the tetrahydrofolate form (H_4folate), making it ready to accept, transport, and donate C1 groups again. This remethylation is also a way to lower

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Table 1. Distribution of Individual Folates Calculated as Folic Acid Equivalents in (A) Some Cereal Products and Yeast and (B) Spinach, Egg Yolk, and Pig Liver Expressed in Percentage of Total Folate

food sample	H_4folate (%)	5-CH_3-H_4folate (%)	5-HCO-H_4folate (%)	10-HCO-H_2folate (%)	10-HCO-folic acid (%)	folic acid (%)	method[a] and source
			(A) Cereal Products and Yeast				
wholemeal wheat flour	16	7	61	nd[b]	16	na[c]	SAX LC-FLD[21]
	6–9	11–23	34–46	*8–12*	16–23	5–10	Affinity LC- FLD/UV[22]
	9.8	10.6	33.7	na	29	17	SPE LC-MS[23]
white bread	5.1	43.4	21.8	na	3.5	26.2	DEAE-Sephadex LC-UV/FLD[24]
	nd	11–22.5	26–29	*19–25*	23–33	4–7.5	Affinity LC-UV/DA[25]
	nd	20–21	16-31	*16–17*	20–21	na	Affinity LC-UV-FLD[26]
	15	44	29	nd	12	na	SAX LC-FLD[21]
	6		nd	na	65.4	nd	Affinity LC-FLD[27]
	32.3	16.7	43.9	na	11.6	na	Affinity LC-MS/MS (SIDA)[27]
	na	80	19	na	na	nd	SPE LC-MS/MS[27]
	2–4	32–35	21–23	*25–29*	12	1–2	Affinity LC-FLD/UV[28]
	nd	33	47	na	20	nd	SPE-LC-MS[23]
	7	47	24	*5*	14	3	SPE-SAX LC-MS-MS/SIDA[29]
dry baker's yeast	19.8	77.3	2.9	na	nd	nd	Affinity LC-UV/DAD[30]
	25.4	74.6	nd	na	nd	nd	SPE LC-FLD[31]
	20.3	61.0	16.4	na	2.3	nd	SPE LC-MS[32]
	20.3	73.5	nd	na	6.2	nd	SPE UPLC-FLD[33]
			(B) Spinach, Egg Yolk, and Pig Liver				
spinach	3.0	70.2	26.8	na	na	na	DEAE-Sephadex LC-UV/FLD[34]
frozen		100	msk	na	msk	nd	SAX-LC-UV/FLD[35]
raw	nd	46	nd	*48*	11	nd	Affinity LC-FLD/UV[26]
fresh, cooked	nd	54	16	*30*	5	nd	Affinity LC-FLD/UV[26]
raw, fresh	nd	80	20	nd	nd	nd	Affinity LC-FLD[27]
raw, fresh	10	86	7	nd	nd	nd	Affinity LC-MS/MS[27] (SIDA)
raw	0–19	76–88	5–34	nd	nd	nd	SPE LC-MS/MS[27]
raw, frozen	nd	78	22	na	nd	nd	SPE LC-MS/MS[36]
raw, fresh	23–33	46–54	19–21	na	1.7–2.1	na	ultrafiltration LC-MS/MS[37]
raw, fresh	24.1	52.1	21.5	na	2.3	nd	SPE LC-FLD/UV[38]
raw, minced	0.6	73	9	*3.5*	13	na	SPE-SAX LC-MS-MS/SIDA[29]
egg yolk	nd	81–87	na	na	~10	3–5	SAX LC-UV/FLD[39]
	nd	86	nd	na	14	na	SPE LC-MS[40]
	nd	83	nd	na	17	nd	SPE UPLC-FLD[33]
liver, pig, raw	31.6	50.6	17.8				DEAE-Sephadex LC-UV/FLD[41]
	77.2	23.8	nd	na	na	nd	SAX LC-UV/FLD[39]
lyophilized pig liver (CRM 487)	27.4	33.4	20.2	na	na	19.1	SPE/SAX LC-MS/MS[42]

[a]DEAE, diethylaminoethyl; SPE, solid phase extraction; SAX, strong anion exchange; LC, liquid chromatography; UV ultraviolet detection; DAD, diode array detection; FLD, fluorescence detection; MS, mass spectrometry; SIDA, standard isotope dilution assay. [b]nd, not detected. [c]na, not analyzed.

homocysteine; increased homocysteine levels have been intensely investigated as a risk factor for cardiovascular diseases.[3,4,19]

The three folate intermediates not participating directly as C1 group donors are 5-CH=NH-H_4folate, 5-HCO-H_4folate, and 5,10-CH^+=H_4folate. Instead, these forms play important roles within the folate metabolism as acceptors and transfers of C1 groups (see Enzymatic interconversions).

OCCURRENCE OF DIETARY FOLATES

Good sources of naturally occurring folates are, for example, green leafy vegetables such as spinach and different varieties of cabbages, as well as legumes, citrus fruits, berries, liver, egg yolk, yeast, and wholegrain cereal products, especially bran and germ.[20] Most data on dietary folates compiled in food databases are quantified as total folate by microbiological assays (MA).[10] During the 1990s more extensive attempts to separate and quantify individual folates in foods started, based on HPLC with UV and/or fluorescence detectors. Later, during the past decade, the mass spectrometric detector became the preferred choice of detector because of its superiority in selectivity and sensitivity.

Table 1 gives examples of individual folate forms (calculated as percentages of total folate content) in some typical foods using chromatographic separation (HPLC with different detectors).[21−42] Although the proportions (percent) of individual folate forms between different studies and food items clearly differ, data are consistent concerning which forms are typically found. As Table 1 clearly shows, the major native folate form in most foods, except cereals and cereal products, is 5-CH_3-H_4folate, ranging between 50 and 90% of the total folate pool. 5-CH_3-H_4folate is also the most common form in body fluids (data not shown).

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The second most common native folate form is 5-HCO-H_4folate followed by H_4-folate. Thereafter, 10-HCO-folic acid is frequently reported. 10-HCO-folic acid is the stable oxidation product of the tetra and dihydro forms of 10-formyl folates. No study reports the presence of 10-HCO-H_4folate in foods, but a few studies report the occurrence of 10-HCO-H_2folate in some foods.[25,26,28,29]

In cereal products, the two formyl folates, 5-HCO-H_4folate and 10-HCO-folic acid, are dominating forms, for instance, in whole grain of wheat and rye and bread made from these flours. The synthetic form, folic acid, can be found in fortified foods. It is stable and easy to quantify. Today, over 60 countries worldwide practice mandatory folic acid fortification of flours from wheat and/or corn as prophylaxis of neural tube defects.[43] Moreover, other food items can be fortified with folic acid according to national regulations.

If we compare the analyzed folate forms listed in Table 1 with those presented in living cells (see Figure 2), we can identify and quantify H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate, and the forticant, folic acid, whereas 10-HCO-H_4folate now is represented by the oxidation products 10-HCO-H_2folate and/or 10-HCO-folic acid. Four of the other folate derivatives occurring in living cells, namely, H_2folate, 5,10-CH^+=H_4folate, 5,10-CH_2-H_4folate, and 5-CH=NH-H_4folate, are generally not recorded in foods. The reasons behind the discrepancies in folate forms present in living cells and the folate forms finally analyzed in foods will be further discussed in the next sections. First, an overview of the stability of folates in terms of enzymatic and chemically driven interconversions will be given.

ENZYMATIC INTERCONVERSIONS OF FORMYL FOLATES

Folate metabolism, briefly summarized above and displayed in Figure 2, also includes many enzymes for which the different folate forms are coenzymes. It is beyond the scope of this review to go into detail in this complex area, where differences in folate metabolism occur between cells (plant cells vs mammalian cells) and within cells (cytoplasm vs mitochondria). There are many reviews on the subject.[1,3,4,16,44] In general, H_4folate can interconvert enzymatically to 5,10-CH_2-H_4folate or to 5-CH=NH-H_4folate by accepting C1 groups from serine or from histidine and purines, respectively. 5-CH=NH-H_4folate interconverts its C1 group enzymatically when forming 5,10-CH^+=H_4folate. As seen in Figure 2, 5,10-CH^+=H_4folate is a sort of "spider in the web", enzymatically in contact with all other folate forms. This form is a precursor to two of the actively C1-donating folate forms, for example, 10-HCO-H_4folate and 5,10-CH_2-H_4folate.

10-HCO-H_4folate can be enzymatically formed from 5,10-CH^+=H_4folate and vice versa by 5,10-methenyltetrahydrofolate cyclohydrolase. The C_8 and C_2 positions in the purine rings of DNA and RNA are derived from 10-HCO-H_4folate in a reaction catalyzed by glycinamide ribonucleotide transformylase and aminoimidazole carboxamide ribonucleotide formyltransferase. The 10-HCO-H_4folate needed for purine biosynthesis can be either directly derived from the 10-formyltetrahydrofolate synthetase-catalyzed reaction of H_4folate or can be derived from the oxidation of 5,10-CH_2-H_4folate via 5,10-CH^+=H_4folate, catalyzed by the NADP-dependent 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methenyltetrahydrofolate cyclohydrolase.[4,18,45]

5,10-CH^+=H_4folate can be enzymatically interconverted to 5-HCO-H_4folate by 5-formyltetrahydrofolate cyclodehydrase. Another enzyme, methenyltetrahydrofolate synthetase, interconverts 5-HCO-H_4folate back to 5,10-CH^+=H_4folate.[45] 5-HCO-H_4folate normally accounts for 3−10% of total intracellular folate in mammalian cells; however, its metabolic function in cells has not been elucidated. It has been suggested to play a regulatory role or serve as a reservoir of C1-carrying folates.[46−49]

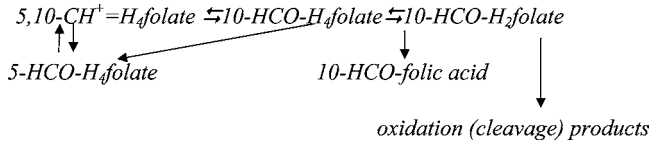
There are no direct enzymatic conversions between 5-HCO-H_4folate and 10-HCO-H_4folate other than via 5,10-CH^+=H_4folate.

5,10-CH^+=H_4folate may also convert to 5,10-CH_2-H_4folate by 5,10-methylenetetrahydrofolate dehydrogenase. 5,10-CH_2-H_4folate is a coenzyme for thymidylate synthase, which involves the addition of a formaldehyde group to deoxyuridine monophosphate (dUMP), leading to the formation of deoxythymidine monophosphate (dTMP) and dihydrofolate (H_2folate) (see Figure 2). Synthesis of deoxynucleotides, which is mediated by thymidylate synthase and ribonucleotide reductase, is considered to be the rate-limiting step in DNA synthesis.[4,18,45]

An important part of C1 use involves the reduction of 5,10-CH_2-H_4folate to 5-CH_3-H_4folate by the enzyme 5,10-methylenetetrahydrofolate reductase. This reaction is physiologically irreversible. The methyl group of 5-CH_3-H_4folate is then donated to homocysteine, leading to methionine synthesis, which is catalyzed by methionine synthase.[4,18,45]

CHEMICAL INTERCONVERSION AND DEGRADATION OF FORMYL FOLATES

In addition to the enzymatic interconversions between different folate forms, briefly summarized above, there are also chemically induced interconversions between some folate forms as well as oxidation/degradation reactions. For formyl folates these reactions can be outlined in the following way:



The fact that reduced formyl folates are interconvertible through the 5,10-CH^+=H_4folate intermediate was recognized already in the early 1950s.[50−52] These reactions are mainly influenced by pH but are also affected by temperature and oxygen/antioxidants as shown in Table 2. Most of the research until 2000 was performed in model systems at ambient temperature by observing increases/decreases in optical densities of absorption spectra at λ_{max} for 5-HCO-H_4folate (282 nm) and/or 10-HCO-H_4folate (258 nm) and/or 5,10-CH^+=H_4folate (355 nm).[13−15,53,54]

Interconversion of 5-HCO-H_4folate at Acidic pH. Formation of 5,10-CH^+=H_4folate occurs by acidification of either 5- or 10-HCO-H_4folate. The reaction is termed dehydration because one molecule of water is lost, leading to cyclization of the molecule. At low to ambient temperature, equilibrium between 5-HCO-H_4folate and 5,10-CH^+=H_4folate is reached after 24 h,[55] whereas heating by boiling increases the rate of the interconversion.[56] The equilibrium is gradually shifted toward 5,10-CH^+=H_4folate, and its formation becomes faster with decreasing pH. Baggott et al.[57] found only small amounts of 5,10-CH^+=H_4folate at pH 4.4 after 20 h at ambient temperature, whereas 5,10-CH^+=H_4folate increased to 72% at pH 3.3 after reaching equilibrium. Only 6% of 5-HCO-H_4folate was retained after heat treatment at pH 2.3 when using antioxidants ascorbic acid (2%) and BAL (0.1%) in combination.[56] According to data in the literature, this conversion is complete and quantitative at pH below 2.[14,58−61] At such low pH, 5,10-CH^+=H_4folate is stable toward oxidation even without antioxidants.[53]

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Table 2. Reaction Products for 5-HCO-H_4folate, 10-HCO-H_4folate, and 5,10-CH^+=H_4folate Depending on pH, Temperature, and Use of Antioxidants

	pH <2	pH 2−4.5		pH >4.5	
compound	low temp (4−23 °C)	low temp (4−23 °C)	heat treatment[a]	low temp (4−23 °C)	heat treatment[a]
5-HCO-H_4folate					
+ antioxidants	5,10-CH^+=H_4folate	5,10-CH^+=H_4folate, 5-HCO-H_4folate	5,10-CH^+=H_4folate, 5-HCO-H_4folate	5-HCO-H_4folate	5-HCO-H_4folate
− antioxidants	5,10-CH^+=H_4folate	5,10-CH^+=H_4folate, 5-HCO-H_4folate, 10-HCO-H_2folate oxidation products	5,10-CH^+=H_4folate, 5-HCO-H_4folate oxidation products	no reports	no reports
refs	14, 58−61	14, 55−57, 59, 65	14, 55−57, 59, 65	56, 59, 65	56, 59, 65
10-HCO-H_4folate					
+ antioxidants	5,10-CH^+=H_4folate	5,10-CH^+=H_4folate	no reports	10-HCO-H_4folate	no reports
− antioxidants	not reported	no reports	no reports	10-HCO-H_2folate 10-HCO-folic acid oxidation by cleavage	5-HCO-H_4folate
refs	50, 58	15, 59		14, 16, 63	53
5,10-CH^+=H_4folate					
+ antioxidants	5,10-CH^+=H_4folate	5-HCO-H_4folate		10-HCO-H_4folate	10-HCO-folic acid
− antioxidants	5,10-CH^+=H_4folate	10-HCO-H_2folate	(11*S*)-5,10-OHCH-H_4folate	10-HCO-H_2folate	no reports
			5-HCO-H_4folate	5-HCO-H_4folate	
refs	14, 58−61	57	61	14, 50, 53, 59, 64	14, 50, 53, 59, 64

[a]Heat treatment >50 °C, boiling 10-60 min.

In general, derivatives substituted at the N-5 position, for example, 5,10-CH^+=H_4folate and 5-HCO-H_4folate, are more stable against oxygen.[1,14,62]

Interconversion of 10-HCO-H_4folate at Acidic pH. Formation of 5,10-CH^+=H_4folate from 10-HCO-H_4folate by acidification follows the same route as 5-HCO-H_4folate, but the rate of its interconversion into 5,10-CH^+=H_4folate is up to 1000-fold faster.[15,55,58,59] Compared with 5-HCO-H_4folate, 10-HCO-H_4folate is more sensitive toward oxidation, almost as sensitive as H_4folate.[14,50,53] Thus, if not protected by antioxidants such as ascorbate or 2-mercaptoethanol, 10-HCO-H_4folate is oxidized to 10-HCO-H_2folate, which can still be easily converted back to the 10-HCO-H_4folate by a change in the redox state such as would be caused by ascorbic acid.[16,53] Further oxidation of reduced 10-HCO-folates gives either the stable form, 10-HCO-folic acid, which still is biologically active, or cleavage of the molecule into biological inactive products.[53,63] Heating or prolonged standing at neutral pH enhances the rate of isomerization of reduced 10-HCO-folates into 5-HCO-H_4folate.[14] Thus, changing pH by acidification from 7 to 2 allows 10-HCO-H_4folate to interconvert either by dehydration to 5,10-CH^+=H_4folate or by oxidation to 10-HCO-H_2folate and 10-HCO-folic acid or to cleavage products. Moreover, heating of 10-HCO-H_4folate can interconvert this form to 5-HCO-H_4folate by isomerization. For all of these interconversions, antioxidants, oxygen, time, and temperature have impacts.

Interconversion of 5,10-CH^+=H_4folate by Increasing pH. As indicated in the reaction shown above, the formation of 5,10-CH^+=H_4folate from either 5- or 10-CHO-H_4folates during acidification is reversible. This reaction may be reversed at pH ≥2. Inconsistent results on conversion products of 5,10-CH^+=H_4folate, especially in the acidic pH range 2.5−5, are found in the literature.[57,60,61]

Stover and Schirch[60,61] studied the interconversion of 5,10-CH^+=H_4folate in the pH range 3−5. In their model system 5,10-CH^+=H_4folate was heated for several hours at 50 °C in closed tubes degassed with argon but without antioxidants. The authors suggested that 5,10-CH^+=H_4folate hydrated in the pH range 3−5 into a stable intermediate, (11*S*)-5,10-OHCH-H_4folate and small amounts of 5-HCO-H_4folate. This intermediate was stable at pH values from 4 to 9 for hours, but at pH values <2 it was reconverted to 5,10-CH^+=H_4folate. At pH values >8 this hydroxyl form was converted back to 5-HCO-H_4folate.

Bagott[57] investigated the interconversion of 5,10-CH^+=H_4folate between pH 2.5 and 4.5. The reaction was studied in ambient temperature for 48 h in the absence and presence of different antioxidants, for example, ascorbate, dithiothreitol (DTT), diethylaminoethyl (EDTA), and mannitol. The interconversion of 5,10-CH^+=H_4folate could be shown to be reversible into 5-HCO-H_4folate at pH 2.5 without antioxidants and also at pH 4.5 in the presence of ascorbate. The fact that the reaction was reversible suggested that the hydrolysis product was only 5-HCO-H_4folate. At pH 4.0−4.5, the hydrolysis gave approximately equal concentrations of 5,10-CH^+=H_4folate and 5-HCO-H_4folate in the presence of antioxidants.

If no antioxidants were used, Baggott[57] found considerable amounts of 10-HCO-H_2folate to be formed at pH 4−4.5. This finding contradicted studies conducted by Stover and Schirch.[60,61] Baggott[57] concluded that it is likely that 10-HCO-H_2folate, which was found to be formed without antioxidants, was misidentified as 5,10-HOCH-H_4folate by Stover and Schirch[60,61] because they did not add antioxidants to their samples either.

Several authors have shown that the interconversion of 5,10-CH^+=H_4folate produces solely 10-HCO-H_4folate at neutral and alkaline pH in the presence of antioxidants, being essentially complete at pH values >7.[14,53,55,60,61,64] Long-term standing or heating with and without sufficient protection by antioxidants interconverts 10-HCO-H_4folate into the more stable 5-HCO-H_4folate and 10-HCO-folic acid and, perhaps, also some cleavage oxidation products, which are biologically inactive.[14]

Mechanisms of Interconversion Reactions. The pH-dependent interconversion of formyl folates through the 5,10-CH^+=H_4folate intermediate has been classified as an acid−base-catalyzed hydrolysis/cyclization/dehydration reaction.[14] Being

acid–base-catalyzed means that the interconversions are more pronounced and rapid at acidic and alkaline pH values, whereas the formyl folates are more stable at slightly acidic, neutral, and weakly alkaline pH values, that is, at pH 5–7. However, because 10-HCO-H_4folate also is sensitive to oxygen and heating, it can interconvert to all folate forms shown in the reaction scheme above.

Although 5-CHO-H_4folate is recognized to be stable toward oxidation and could be lost only through interconversion to 5,10-CH^+=H_4folate at acidic pH, a recent study found pronounced effects of antioxidants.[56] These authors compared the stability of 5-HCO-H_4folate and 5,10-CH^+=H_4folate formed from 5-HCO-H_4folate at pH 2.3 with and without antioxidants at room temperature and after heat treatment. The retention of both 5-HCO-H_4folate and 5,10-CH^+=H_4folate formed by interconversion at pH 2.3 was found to be much lower without antioxidants; heating enhanced the losses of both compounds further. It was suggested that it was not possible to achieve the equilibrium in the interconversion reaction between 5-HCO-H_4folate and 5,10-CH^+=H_4folate if no antioxidants were used to protect folates from oxidation because of simultaneous formation of 10-HCO-H_4folate from 5,10-CH^+=H_4folate and its further oxidation. In fact, the equilibrium could be constantly shifting to the right, that is, formation of 5,10-CH^+=H_4folate, which was removed due to formation of 10-HCO-H_4folate, which in turn was removed due to oxidation reactions.

INFLUENCE OF INTERCONVERSION AND THERMAL DEGRADATION ON FORMYL FORMS PRESENT IN FOODS

Clearly, as long as the endogenous enzymes are still active in the food sample, for instance, in raw or fresh foods or foods containing living microorganisms, for example, lactic acid producing bacteria (LAB) or yeast (in dough), or during sprouting or malting, completely unforeseen interconversions between different folates forms may occur. The extent to which enzyme-driven interconversions occur depends on cell type, animal versus plant cells, and whether the cells are kept intact or not. Intact cells can provide better stabilization of formyl folates by being bound to enzymes or other proteins. Living cells, for example, fresh plant foods or added living microorganisms such as yeast or lactic acid producing bacteria, can keep their cells intact for a long time. Such cells can continue to biosynthesize different folate forms.[66]

In addition, some foods have a natural acidic pH: fruits and berries rich in organic acids such as cranberry and orange juice (pH 2.6–3.5) or foods becoming acidic due to processing such as fermentation and pickling (pH 3.5–4.5). The entire period elapsing from harvest/slaughter until sampling of such food items after processing and distribution may be long and lead to significant interconversions, particularly when cell integrity is compromised. Interconversions of formyl folates occur for several reasons, not only because of the low pH or the presence of endogenous enzymes but also because of thermal treatment or oxidation by oxygen, especially in the presence of pro-oxidants such as Fe and Cu ions. There are very few studies focusing on the fate and interconversions of the formyl folates during processing and storage.

Indeed, it is important to understand that folate forms present in the final food extracts may differ considerably from those occurring in vivo. Most studies investigating individual folate forms in food samples include the following five folate forms: folic acid, H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate, and 10-HCO-folic acid. On the basis of what we know about chemical interconversions of the formyl folates, we can expect that 5-HCO-H_4folate can be decreased by interconversion to 5,10-CH^+=H_4folate in acidic foods. Because 5,10-CH^+=H_4folate can also reversibly form 10-HCO-H_2folates or 10-HCO-H_4folates, this conversion can increase the losses of the originally present 5-HCO-H_4folate further, especially if the formed 10-HCO-H_2folates or 10-HCO-H_4folates are unprotected by antioxidants. On the other hand, simultaneous isomerization of reduced 10-HCO-H_4folates can lead to increasing levels of 5-HCO-H_4folate. Moreover, some loss of reduced 10-formyl-folates might occur by oxidative cleavage. The stable 10-HCO-folic acid, on the other hand, most probably corresponds to that part of the originally present 10-HCO-H_4folate that has been oxidized and not been interconverted to 5-HCO-H_4folate or 5,10-CH^+=H_4folate or cleaved into oxidation products. A certain part might also come from the reversible reaction of 5,10-CH^+=H_4folate into 10-HCO-H_2folates or 10-HCO-H_4folates. The extent of these conversions is dependent on the pH, time, temperature, and presence/absence of natural antioxidants.

Because parallel enzymatic and chemical interconversions of formyl folates occur in foods during their way from harvest/slaughter, through processing, storage, distribution, and cooking until consumption, a gap between originally present and actually found formyl folates is to be expected. As described in the next section, analytical methods for the determination of food folates include changes in pH, temperature, and antioxidants, which might add to further interconversions of the formyl folates.

DETERMINATION OF FORMYL FOLATES IN FOODS BY HPLC AND THEIR INTERCONVERSIONS DURING ANALYTICAL PROCEDURE

Interconversions and stabilities of formyl folates cannot be studied by microbiological assay (MA) because *Lactobacillus casei/rhamnosus* responds to all native bioactive folate forms including biologically active interconversion products. Hence, MA gives the sum of all forms, that is, total folate, except different racemic forms or polyglutamate forms. There are standard methods assessed in collaborative studies established for microbiological assays, for fortified infant formula,[67] cereals,[68] and foodstuffs.[69] A disadvantage of the microbiological assay is its inability to distinguish between individual folate forms and overall time needed for analysis, almost 2 days, which emphasizes the need of optimal protection by antioxidants.

In contrast, HPLC methods that separate and analyze individual folate forms can be conducted within much shorter time (approximately 4–6 h). Yet, there are no standard methods established for chromatographic determination of food folates. This is not surprising given the challenging task of optimizing and validating the methodology to include all native folate forms of which most are unstable and occur in very low amounts (μg level per 100 g).

Determination of individual folate forms by high-performance liquid chromatography (HPLC) includes three main steps: (i) extraction and deconjugation; (ii) cleanup/concentration; and (iii) detection/quantification, as outlined in Figure 3. Crucial for the final result is the use of proper antioxidants and pH to protect the labile forms throughout the procedure. Moreover, cautions have to be taken to reduce the UV light and oxygen (by degassing with nitrogen or argon).

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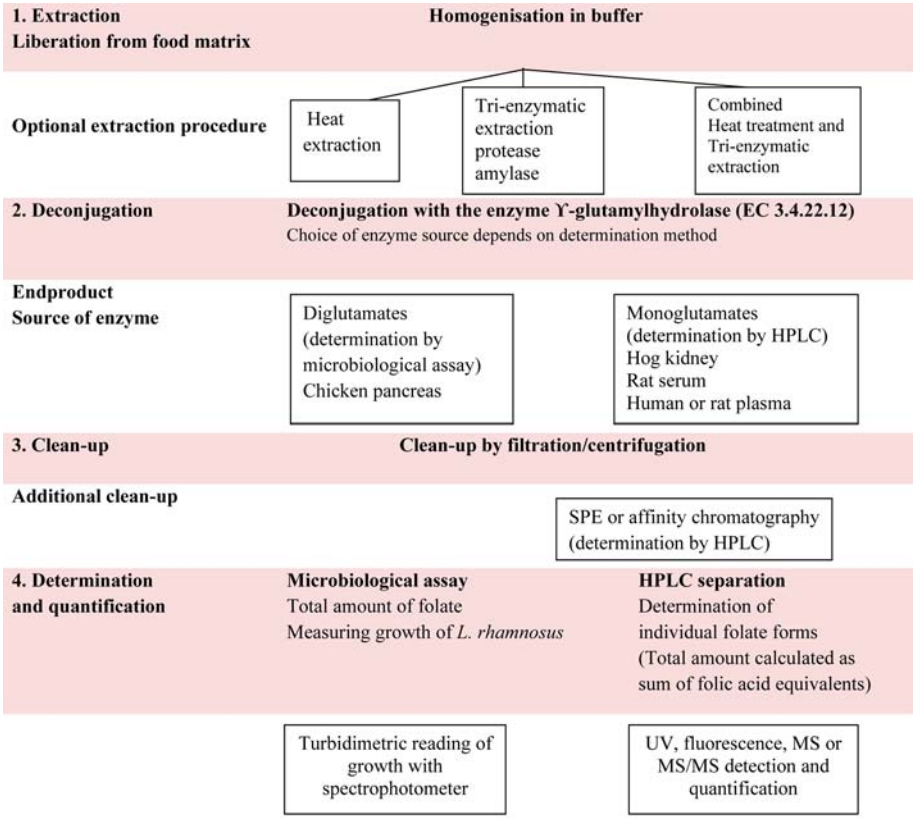

Figure 3. Schematic overview of analytical methods for folate determination modified from refs 15,40, and 63.

Table 3 summarizes the analytical conditions used in HPLC analyses for food folates by the research groups analyzing individual food folates during the past 10−15 years (for references, see Table 3).

Extraction. Most studies use neutral pH during extraction and deconjugation, which should mean negligible conversion of 5-HCO-H_4folate and 10-HCO-H_4folate into 5,10-CH^+=H_4folate. Extraction and deconjugation could include several enzymatic treatments for some hours followed by repeated enzymatic inactivations by short-time (5 min) boiling of the samples. This leads to interconversion of 10-HCO-H_4folate into 5-HCO-H_4folate because heating enhances this interconversion. In parallel, 10-HCO-H_4folate can be oxidized, to either 10-HCO-H_2folate or further to 10-HCO-folic acid, and/or cleaved into inactive products, depending on how efficiently the reduced 10-formyl folates are protected by antioxidants.

Cleanup by SPE or Affinity Chromatography. In the cleanup procedure including the purification either by affinity chromatography or by solid phase extraction (SPE), the folates are eluted by acidic buffers. From Table 3 it is clear that affinity chromatography causes the most pronounced acidification by operating close to pH 2 during the 10−20 min the elution takes. This should lead to gradual interconversion of reduced 5- or 10-formyl folates into 5,10-CH^+=H_4folate. Because, the eluates are collected in alkaline buffers containing antioxidants, the interconversion turns back mainly to 5-HCO-H_4folate; however, some amount of 5,10-CH^+=H_4folate might be interconverted to 10-HCO-H_4folate and further oxidized if antioxidant protection is insufficient. During the cleanup procedure for the formyl folates, affinity chromatography must be carefully done due to lower affinities between formyl folates and folate binding proteins (FBP). It is therefore important to estimate the optimal loading capacity of the column capacity for food samples containing a mix of folate forms to achieve acceptable yields for the formyl folates.[25,70,71] Moreover, no studies seem to have been reported on the binding capacity of FBP toward 10-HCO-folates of different oxidation states. Hence, the FBP-based affinity chromatography should be used with caution for the analysis of foods containing formyl folates.[72]

As seen in Table 3, SPE-based cleanup procedures using strong-anion exchange (SAX) cartridges elute the folates by sodium acetate solution containing sodium chloride salt (5 or 10%) and ascorbic acid (1%), which keeps the pH close to 5 (for references, see Table 3). Therefore, there might be negligible pH-induced interconversions. However, it is still important to use an optimal antioxidant protection to prevent oxidation and degradation of formyl folates. Worth mentioning is also that SAX is not always sufficiently selective for 5-HCO-H_4folate and 10-HCO-folic acid,[73] due to interfering impurities in the sample matrix that coelute in both SAX and HPLC systems, leading to noisy chromatograms with a possible masking of the peak(s) of interest[35,74,75]

Separation by HPLC. After cleanup by affinity chromatography or solid phase extraction, the eluted samples are either frozen until HPLC analysis or directly put into a thermostated autosampler (often below 10 °C). If the sample sequence contains more than 30 samples, up to 24 h might elapse in the autosampler prior to quantification of the last samples. The extent to which acid-catalyzed interconversions of formyl folates take place under these conditions (low temperature/long time) has been little studied. One recent study by Kirsch et al.[76] can be illustrative. They studied the stability of individual folates stored at 4 °C during 1.5 and 24 h. Aqueous solutions of folate standards were used in buffers of different

Table 3. Buffers and pH Conditions for Determination of Formyl and Other Folate Forms in Foods by Different Liquid Chromatographic Methods

extraction buffer[a]	pH	enzyme treatment[b]	pH	cleanup method,[c] elution buffer[d]	pH	mobile phase[e]	detection[f] and source
CHES−HEPES	7.85	TE/RP	7.8	Affinity (FBP), TFA	2.2[g]	PB/ACN	UV/DAD[25]
CHES−HEPES	7.85	TE/RP	7.0	Affinity (FBP), TFA	2.2[g]	PB/ACN	UV/FLD[26]
CHES−HEPES	7.85	TE/HK	4.9	Affinity (FBP), TFA	2.2[g]	PB/ACN	UV/FLD[28]
CHES−HEPES	7.9	RP	7.9	Affinity (FBP), PB	2.5	PB/ACN	ECD[80,81,88]
PB	8−9	DE/RP	6.6	SPE-SAX, NaAc/-PB	4.5	PB/MeOH post-column, PB	FLD[21]
CHES−HEPES	7.85	DE/RP	7.5	Affinity (FBP), TFA	2.2[g]	FA/ACN	MS/MS-SIDA[27]
				SPE-SAX, Ac/NaCl	5.0	FA/ACN	FLD[27]
CHES−HEPES	7.85	TE/CP	7	SPE-SAX, Ac/NaCl	5.0	FA/ACN	MS/MS-SIDA[36]
MES	5	TE/CP + RP	5	SPE-SAX, Ac/NaCl	5.0	HAc/FA/ACN	MS/MS-SIDA[84]
PB	6.1	ME, RP	6	SPE-SAX, Ac/NaCl	5.0	HAc/ACN	MS[23,32]
PB	6	TE/RP	6	SPE-SAX, PB/NaCl	6	FA/ACN	MS/MS[85−87]
PB	6.5	ME, RP	6.5	ultra-/MP-filtering	6.5	FA/ACN	MS/MS[37]
PB	7.2	TE/RP	7.5	ultra-/MP-filtering	7.5	FA/ACN	MS/MS (IS)[82]
PB	7.2	TE/RP	7.5	ultra-/MP-filtering	7.5	FA/ACN	UPLC-MS/MS[83]
PB	6.5	ME, RP	6.5	SPE-SAX, Ac/NaCl	5.0	PB/ACN	FLD/UV[38]
CHES−HEPES	7.85	DE/RP	6.1−7.2	SPE-SAX, Ac/NaCl	5.0−2.2[h]	FA/ACN	LC-MS/MS[42]
PB	6.1	ME, RP	6	SPE-SAX, Ac/NaCl	5.0	PB/ACN	UV-DAD, FL[33]

[a]CHES−HEPES, 2-(*N*-cyclohexylamino)ethanesulfonic acid−*N*-(2-hydroxyethyl)-piperizine-*N*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PB, phosphate buffer. [b]TE, trienzyme; DE, dienzyme; ME, monoenzyme, RP, rat plasma conjugase, CP, chicken pancreas conjugase, HK, hog kidney conjugase. [c]FBP, folate binding protein. [d]TFA, trifluoroacetic acid; SPE, solid phase extraction; SAX, strong anion exchange; Ac, acetate. [e]FA, formic acid; ACN, acetonitrile. [f]LC, liquid chromatography; UPLC, ultrahigh-pressure liquid chromatography; UV, ultraviolet; DAD, diode array detector; FLD, fluorescence detector; ECD, electrochemical detector; MS, mass spectrometry; SIDA, standard isotope dilution assay. [g]Eluate collected in alkaline. [h]Samples stored in FA/ACN.

pH values (2.6, 3.4, and 7.0) containing 0.1% ascorbic acid. The main interconversion products were presented for each folate form on the basis of UPLC-MS/MS analysis. Indeed, 5-HCO-H_4folate converted under acidic conditions to 5,10-CH^+=H_4folate; ∼25% at pH 3.4 and ∼51.5% at pH 2.6 after 24 h.

Once the purified samples are injected onto the chromatographic column, the individual folate forms separate from each other and antioxidants originally present in the samples. Mobile phases consisting of either acetonitrile or methanol mixed with acidic buffers (pH 2.2−3.4) such as phosphoric acid or phosphate buffer or formic or acetic acid are used for separation. Note that no antioxidants are added to the mobile phase, which makes folates unprotected against oxidation. Moreover, column temperatures might vary between 20 and 35 °C (for references, see Table 3). The extent to which acid-catalyzed interconversions of formyl folates occur at this stage of analysis has received little attention over the years. Recently, Jastrebova et al.[56] used a flow injection model to investigate on-column interconversions. Flow injection analysis was performed for 5-HCO-H_4folate at two concentrations (0.5 and 5 μg/mL) in two different buffers (formic acid, pH 2.9; and acetic acid, pH 3.4) and at simulated column temperatures (15, 20, and 35 °C). The lowest retention (70%) was found for 0.5 μg/mL 5-HCO-H_4folate at 35 °C in formic acid (pH 2.9). The use of pH >3 and lower column temperature provided better stability of 5-HCO-H_4folate.

5,10-CH^+=H_4folate in Foods, an Artifact? It is clear that chromatographic analysis of folates might induce interconversions as well as oxidations of formyl folates, especially during the last step of the analysis. Analytical chemists need to either take actions against pH-induced conversions of formyl folates or include 5,10-CH^+=H_4folate among the standards. Whereas this problem seems to be considered by researchers analyzing clinical samples,[77−79] pH-driven interconversions of formyl folates during determination of food folates have received little attention so far. Studies on folate biofortification of tomato fruit reported 20−40% of total folate as 5,10-CH^+=H_4folate.[80,81] These authors used affinity chromatography and HPLC with electrochemical detection, both steps conducted at approximately pH 2.5 (Table 3). The authors concluded that 5,10-CH^+=H_4folate was a result of interconversion of 10-HCO-H_4-folate during analysis, without noticing that parts of 5-HCO-H_4folate could also have been interconverted, although their interconversion takes place more slowly in comparison with that of 10-HCO-H_4-folate.

We have found three other studies reporting occurrences of 5,10-CH^+=H_4folate in food samples.[29,82,83] Two of these studies reported wild-type rice to contain 20−30% formyl folates (5-HCO-H_4folate and 10-HCO-folic acid) and 6−10% 5,10-CH^+=H_4folate. The analyses were performed by LC-MS/MS or UPLC-MS/MS after purification by ultrafiltering and using formic acid (0.1%)/acetonitrile as mobile phase. Because the analysis was done at neutral pH during all steps, except in the quantification step when formic acid (0.1%) was used as mobile phase, it is most probable that 5,10-CH^+=H_4folate was formed after the samples were injected on the column (60 °C) and became acidic below pH 3 by the mobile phase.[82,83] The third study[29] reported 5,10-CH^+=H_4folate in concentrations mostly below 1% in about 15 different food items. All three studies reported low absolute recoveries of 5,10-CH^+=H_4folate, 50−60 and 23%, respectively. Moreover, the 5,10-CH^+=H_4folate stock solution was prepared at pH 3 and 5, respectively, which may cause interconversions into 5- and 10-formylfolates as listed in Table 2. As mentioned earlier, 5,10-CH^+=H_4folate is stable only below pH 2.

Detection of 10-HCO-H_2folate in Foods. As discussed in previous sections, data on the occurrence of formyl folates in

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foods reported in the literature usually include only two formyl folate forms, 5-HCO-H_4folate and 10-HCO-folic acid. Reduced 10-formyl folates, for example, 10-HCO-H_4folate and 10-HCO-H_2folate, are seldom reported. However, previously 10-HCO-H_2folate was reported to occur in bread[25,26,28] as well as in some vegetables.[26] This work had in common cleanup by affinity chromatography combined with HPLC-FLD and/or UV-DAD; that is, the identification of 10-HCO-H_2folate was less selective than by mass spectrometry. In a recent study,[29] however, Ringling and Rychlik determined 10-HCO-H_2folate in about 15 different food samples, mostly in concentrations in the lower range of 0.2−19%, using LC-MS/MS and stable isotope dilution assay (SIDA). This paper succeeded in analyzing seven different food folate forms. Indeed, this technique is the most advanced and selective method to analyze native folates in food samples. An advantage of using isotope-labeled internal standards added to the food sample prior to extraction is that the standards interconvert in parallel with the native folate forms, thereby achieving relative recoveries exceeding 90%. The absolute recovery, on the other hand, might often be considerably lower.[29,82,83] Currently, very few food folate analysts can afford to use native stable isotopes of all folate vitamers as internal standards.

Because formyl folates in foods might be oxidized and/or reversibly interconverted to other forms during analysis, the analytical results will not accurately reflect the true forms and proportions of formyl folates originally present in food samples. We therefore suggest that formyl folates in foods should be expressed as the sum of 5-HCO-H_4folate, 10-HCO-folic acid, and 5,10-CH^+=H_4folate calculated as folic acid equivalents. 5,10-CH^+=H_4folate is important to include as reference when using mobile phases with pH <3; otherwise, the formyl folates might be underestimated. The inclusion of reduced forms of 10-formyl folates in the assay is questionable, due to their 1000-fold higher speed of interconversion compared with 5-HCO-H_4folate at acidic pH. Moreover, it is important to consider that the standard solutions of 5-HCO-H_4folate and 5,10-CH^+=H_4folate cannot be prepared in the same buffer because these folate forms require different pH values for their optimal stability: neutral or close to neutral for reduced 5- or 10-formyl folates and highly acidic (pH <2) for 5,10-CH^+=H_4folate, to prevent interconversion reactions.

Interconversion phenomena of formyl folates might be a significant contributor behind losses of folate along the food chain as well as during analysis when acidic buffers are used. At present, the literature offers few data on interconversion phenomena either in the food handling chain or during the analytical procedure. As shown in the present review, most knowledge on the stability and interconversion of formyl folates was acquired long ago, using simplified model systems. Indeed, the spread and fragmentary nature of the knowledge on interconversions of formyl folate, here summarized, need to be systematically updated to minimize or avoid the losses of formyl folates as much as possible.

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Notes

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