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Folate content in sea buckthorn berries and related products (*Hippophaë rhamnoides* L. ssp. *rhamnoides*): Determination of folate vitamer stability influenced by processing and storage assessed by stable isotope dilution assay

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Folate Vitamer Stability in Sea Buckthorn

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Abstract

A stable isotope dilution assay for quantitation of folate vitamers in sea buckthorn berries, juice and concentrate using four-fold labeled folate isotopologues of the folate derivatives as the internal standards was adopted using reversed phase liquid chromatography – tandem

mass spectrometry with electrospray ionization. Processing effects and storage stability were investigated during juice and concentrate production from sea buckthorn berries (*Hippophaë rhamnoides*). The technological processing of the berries caused a total degradation of tetrahydrofolate and 5-formyltetrahydrofolate in the generated juice. The content of the main folate vitamer 5-methyltetrahydrofolate remained approximately unchanged during the whole processing from the berries to the concentrate. Sea buckthorn juice was stored under two household storage conditions (6 °C, 25 °C), and also at accelerated aging conditions (40 °C) for up to seven days to determine the effects of storage temperature on the stability of 5-methyltetrahydrofolate. The content of 5-methyltetrahydrofolate was nearly unchanged during the storage at 6 °C after seven days. The juice showed almost identical degradation of 5-methyltetrahydrofolate of about 17-20% at 25 °C and 40 °C after seven days of storage.

Keywords Folate, stable isotope dilution assay, LC-ESI-MS/MS, *Hippophaë rhamnoides,* Sea buckthorn products, Process and storage stability

Introduction

Sea buckthorn (*Hippophaë rhamnoides* L. ssp. *rhamnoides*) is a plant of the family *Elaeagnaceae,* naturally distributed over Asia and Europe. The berries of *Hippophaë*

rhamnoides are rich in flavonoids, carotenoids and vitamins and are traditionally used for ethnomedicinal remedies in Tibet, Mongolia, China and Central Asia. The high nutritive value of sea buckthorn berries and related products has attracted increasing interest also in Europe and North America [1]. Acidum folicum (folic acid, pteroylmonoglutamic acid) is officially listed in the European Pharmacopoeia [2] and is described as an accurately defined water-soluble B vitamin. The folates represent a class of heterocyclic compounds based on the 4-[(pteridin-6-yl methyl)amino] benzoic acid structure differing by their oxidation states, their one-carbon substituents, and by the number of glutamate residues attached [3]. In contrast to folic acid in the natural physiological form of the vitamin, the pteridine ring is reduced to give either 7,8-dihydrofolate or 5,6,7,8-tetrahydrofolate. An outstanding structural characteristic of tetrahydrofolate is the stereochemical orientation at the C-6 asymmetric carbon of the pteridine ring. Solely the 6*S* stereoisomer is biologically active and synthesized in nature. The folate vitamers exist predominantly as polyglutamate derivatives containing from five to seven glutamate residues in γ-peptide linkage [4]. The reduced folate vitamers function as cofactors in single-carbon transfer reactions in the metabolism of nucleic and amino acids [5]. Various studies over the last decades have shown that folates are supposed to reduce the risk of neural tube defects [6], neurological and neuropsychiatric disorders e.g. Alzheimer disease [7] and schizophrenia [8], cardiovascular and haematological diseases [9, 10] and different forms of cancer [11, 12]. Moreover, antioxidant activity of folates has also been discussed [13].

The Recommended Dietary Allowance for women and men is set at 400 µg/day of dietary folate equivalents [14]. Several studies have shown that fruits and berries are one of the main folate sources providing about 15% of the daily folate intake [15, 16]. Obviously, fresh sea buckthorn berries may represent a beneficial addition for the achievement of the Recommended Dietary Allowance of folate [16]. However, commercial production of sea buckthorn juice and concentrate includes various technological separation steps, a high-

temperature-short-time process (HTST) before aseptic filling and the juice concentrate is obtained during thermovacuum evaporation (five stage evaporator, 80–85 °C). Due to the separating and heating process, a degradation of folate vitamers in sea buckthorn juice has to be expected. Therefore, an assessment of the biological activity of the folate vitamers in juice and juice concentrate is required.

Determination and quantitation of total folates has commonly been performed by microbiological assays based on the turbidimetric measurement of bacterial growth of *Lactobacillus casei* [17, 18]. This sensitive method responds to nearly all reduced and oxidized folate derivatives. However, this technique cannot distinguish between the individual folates. Due to the different stability of the folate vitamers, the characterization of the folate derivatives is indispensable for an accurate assessment of the influences of storage and processing [19]. Slight differences in the acidic and hydrophilic character of the folates allow the determination of the native folate derivative patterns by high performance liquid chromatography (HPLC). A versatile and accurate methodology for determination in food matrices is the HPLC-technique coupled to mass spectrometry (MS) with electrospray ionisation (ESI) using a stable isotope dilution assay (SIDA) [20-24]. The SIDA procedure employing four-fold labeled isotopologues of five different folate vitamers (stable isotope: ^{2}H) as internal standards [21] (Fig. 1) ensures that interferences of food matrices and different vitamer stabilities have no influence on the results of quantitation [21-24].

In the past, in a limited number of studies the effects of processing and of storage on folate stability have been assessed with differentiation of the single vitamers [25, 26]. Until now, sea buckthorn berries and related products such as juice and concentrate were not studied regarding their storage stability and the influence of processing effects on the folate content. Previous studies investigated the effects of processing on the organic acid and soluble sugar content, vitamin C, vitamin K_1 and pantothenic acid in sea buckthorn juices and concentrates [27-29]. The primary objective of the present study was to survey the effects of processing on

stability of different folate vitamers in sea buckthorn juice and concentrate. A second aim was to assess the influence of consumer storage conditions, and also accelerated aging conditions in reducing the content of folate derivatives in sea buckthorn juice.

Experimental

Chemicals

The following chemicals were obtained commercially from the sources given in parentheses: acetonitrile, formic acid, KH_2PO_4, 2-mercapto ethanol, methanol, Na_2HPO_4, sodium acetate, (Merck, Darmstadt, Germany), α-amylase Type II-A from *Bacillus* spp., MES, protease Type XIV from *Streptomyces*, ascorbic acid (Sigma, Deisenhofen, Germany), pteroyl triglutamate (Schircks, Jona, Switzerland).

[$^2H_4$]-5-Methyltetrahydrofolate, [$^2H_4$]-5-formyltetrahydrofolate, [$^2H_4$]-10-formylfolate, [$^2H_4$]-tetrahydrofolate and [$^2H_4$]-folic acid were synthesized as reported recently (Fig. 1) [21].

The extraction buffer consisted of aqueous 4-morpholineethanesulfonic acid (0.2 mol/L) at pH 5.0 contained ascorbic acid (2%) and 2-mercapto ethanol (20 mmol/L).

The conditioning buffer for solid phase SAX cartridges (Bakerbond, quaternary amine, 500 mg, No. 7091-3, Baker, Gross-Gerau, Germany) was prepared by mixing aqueous solutions of Na_2HPO_4 (0.01 mol/L, 62 mL), of KH_2PO_4 (0.01 mol/L, 28 mL), and 2-mercapto ethanol (0.2 mL), adjusting the mixture to pH 7.5 and finally making it up to 100 mL with water.

Chicken pancreas solution was prepared by dissolving 5 mg chicken pancreas (DIFCO, USA) in phosphate buffer (30 mL, 0.1 mol/L, pH 7.0) containing 1% ascorbic acid. Water for buffers and HPLC was purified by a Milli-Q-system (Millipore GmbH, Schwalbach, Germany).

160

161 Food samples

162

163 Two berry varieties of *Hippophaë rhamnoides* were collected. The smaller berry variety was
164 harvested in southern Germany (Area 1) and the second berry variety in Romania (Area 2)
165 from commercial plantings in September 2006. The samples were stored at –20 °C after
166 packing.

167

168 Processing of sea buckthorn juice (Area 1, Area 2)

169

170 The frozen berries were preheated to 8 – 12 °C before mashing. The mash was subjected to a
171 treatment with pectolytic enzymes for 1 – 2 h at 52 °C and separated into juice and pomace by
172 a decanter machine. The turbid juice product, highly concentrated in pulp and oil was clarified
173 by a plate separator. Before aseptic filling the juice was treated in a high-temperature-short-
174 time (HTST: 90 °C, 45 s) process and rechilled immediately. The juice samples were stored at
175 –20 °C after packing.

176

177 Processing of sea buckthorn juice concentrate (Area 2)

178

179 For production of sea buckthorn concentrate the juice was clarified with bentonite (8–12 h,
180 10–12 °C). After filtration with diatomaceous earth under vacuum, the clear juice was
181 concentrated by thermovacuum evaporation (five stage evaporator, 80–85 °C). The °Brix
182 value was adjusted to 65 for clear juice concentrates. Before aseptic filling the concentrate
183 was treated in a HTST (90 °C, 45 S) process and rechilled immediately. The concentrate
184 samples were stored at –20 °C after packing. Solely concentrate from Area 2 was available
185 from the producer for analysis.

186

187 Storage experiments

188

189 Sea buckthorn juice (Area 1) was weighed (20 ± 0.02 g) into Falcon tubes (50 mL) and
190 capped with screw tops. The tubes were placed separately in two climatic test chambers (HC
191 4055, HC 7057) (Heraeus/ Vötsch, Balingen, Germany). Folate degradation of sea buckthorn
192 juice (Area 1) was studied at 6 °C, 25 °C, and 40 °C for 7 days. The folate vitamers were
193 analyzed after 72 h and then in continuation after 48 h intervals. For sampling at different
194 time sets, individual tubes were always used for investigation. Storage experiments were
195 carried out in the dark.

196

197 Extraction for folate quantitation

198

199 Sea buckthorn berries were homogenized by freezing and grinding in liquid nitrogen.
200 Aliquots of homogenates or juices (0.5 g) were overlaid with 10 mL of extraction buffer
201 containing [$^2H_4$]-5-methyltetrahydrofolate, [$^2H_4$]-5-formyltetrahydrofolate, [$^2H_4$]-10-
202 formylfolate, [$^2H_4$]-tetrahydrofolate, and [$^2H_4$]-folic acid (50-150 ng each). The extractions
203 and analyses were carried out in duplicates from single tubes.

204

205 Deconjugation and subsequent clean-up by strong anion exchange

206

207 Sample suspensions were incubated with amylase (0.03 g) for 2 h at 37 °C and with bacterial
208 protease (0.1 g) for 4 h at 37 °C. After enzyme digestion, the samples were heated at 100 °C
209 for 10 min, cooled on ice and spiked with rat serum (150 µL) and chicken pancreas solution

(2 mL). The deconjugation was performed at 37 °C overnight. For testing conjugase activity, pteroyl triglutamate (1 µg) was added to food extracts prior to deconjugation.

At the end of the conjugase treatment, the extracts were centrifuged (15 min, 13200 rpm) and passed through a syringe filter (0.4 µm, Millipore, Bedford, MA, USA). Subsequently, the complete extracts were subjected to clean-up by solid phase extraction according to Freisleben et al. [22], using Bakerbond SAX cartridges (quaternary amine, 500 mg, No. 7091-3, Baker, Gross-Gerau, Germany). The cartridges were successively activated with two volumes of hexane, methanol, and water, and then conditioned with three volumes of conditioning buffer. After applying the sample extracts, the columns were washed with three volumes of conditioning buffer, and the folates were eluted with 2 mL of aqueous sodium chloride (5%), containing 1% ascorbic acid and 0.1 mol/L sodium acetate. The purified extracts were then subjected to LC-ESI-MS/MS. Each food sample was analyzed in duplicate. Due to the use of isotopologically labeled standards, absolute recovery data were not considered for calculation of folate contents. The purified extracts were stored at –30°C until analysis.

Enzymes were tested for endogenous content of folates and revealed to contain only minuscule amounts of 5-methyltetrahydrofolic acid. These minor contents were subtracted from the folate content determined in the deconjugated extracts.

Folate determination by LC-ESI-MS/MS

The samples (10 µL) were chromatographed on a 250 x 3 mm i.d.; 5 µm, Nucleosil 100-5 C_{18} reversed phase column (Macherey & Nagel, Düren, Germany) in connection to a photo diode array detector and a TSQ quantum triple quadrupole mass spectrometer (Finnigan MAT, Bremen, Germany).

The mobile phase consisted of variable mixtures of 0.1% aqueous formic acid (eluent A) and acetonitrile acidified with 0.1% formic acid (eluent B) at a flow of 0.3 mL/min. Gradient elution started at 100% A raising the concentration of B linearly to 10% within 2 min, raising to 25% within further 23 min, and followed to 100% in 2 min. Subsequently, the mobile phase was held at 100% B for 3 min before equilibrating the column for 15 min at the initial gradient conditions.

During the first 4.5 min of the gradient program, the column effluent was diverted to the waste reservoir. The spectrometer was operated in the positive electrospray mode using selected-reaction monitoring (SRM) [24]. Spray voltage was set to 4000 V, sheath gas pressure was 50 mTorr and auxiliary gas pressure 10 mTorr. Capillary temperature was 330 °C and capillary offset 35 V. Source CID (collision induced dissociation) was used with the collision energy set at 10 V.

Calculation of folate contents and validation data

For each folate vitamer, solutions of unlabeled and labeled compound were mixed in nine mass ratios ranging from 0.06 to 16. The curve revealed a linear response of the peak area ratios to the mass ratios of unlabeled to labeled compound [22].

Subsequent additional experiments revealed detection limits of 1.5, 0.5, 1.2, 0.6, 2.6 µg/100 g fresh weight and quantitation limits of 4.4, 1.5, 3.5, 1.9, 7.7 µg/100 g fresh weight for tetrahydrofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formylfolate and pteroylglutamic acid, respectively [22].

257 Inter-assay precision was determined by repeatedly extracting aliquots of sea buckthorn juice
258 as detailed before. For n = 4 determinations, the relative standard deviation for the single
259 vitamers ranged from 4.7 to 16.8%.
260 The recoveries obtained during the determination of the quantitation limits ranged from 80 to
261 110% depending on the individual vitamers.
262 The content of total folates was calculated as the sum of vitamers each referring to the molar
263 mass of folic acid.
264

Results and discussion

Stable isotope dilution assays of folates

Due to their occurrence in trace amounts, their lability, and also the high variety of vitamers, analysis of folates requires several precautions to obtain accurate data. By using stable isotopically labeled analogues of the folate vitamers as internal standards to correct for losses the lability can be overcome and by using enzymatic deconjugation to transfer polyglutamates to their monoglutamic forms it is possible to analyze all important vitamers. To enhance sensitivity, the extraction solvent and all subsequent solutions contained ascorbic acid and 2-mercapto ethanol for maximum stability of folates. Deconjugation by treatment with rat plasma and a preparation of chicken pancreas converted polyglutamic vitamers to the respective monoglutames, which were then detectable by LC-ESI-MS/MS. Sample cleanup was performed subsequently on strong anion exchange cartridges, which resulted in the LC-ESI-MS/MS chromatograms virtually devoid from matrix interferences as displayed in Fig. 2.

Folate vitamers content in sea buckthorn berries, juice, and concentrate

Total folate contents of sea buckthorn berries and juices from Area 1 and Area 2 analyzed by SIDA ranged from 29 µg/100g up to 82 µg/100 g. The amounts of total folate in berries and juices from Area 2 were 56% and 63% lower than in Area 1, respectively. When comparing these results with our data of pantothenic acid and vitamin K_1 content in sea buckthorn berries and juices [28, 29], we recognized again that berries from Area 1 (smaller berry variety) and the obtained juice showed higher concentrations of these vitamins than those from Area 2. The variation of total folate content between the growing areas of sea buckthorn berries may originate from the cultivar differences and growing conditions [26]. Total folate content of

291 diluted juice concentrate (1:6) was measured at 28 µg/100 g. The major folate derivative in
292 sea buckthorn berries was analyzed as 5-methyltetrahydrofolate with an amount of 31 µg/100
293 g (Area 1) and 68 µg/100 g (Area 2). The concentration of tetrahydrofolate comprised 14% of
294 the total folate content (berries from Area 2) and was below the limit of detection (< 1.5
295 µg/100 g) in berries from Area 1. Remarkable amounts of 5-formyltetrahydrofolate of 16
296 µg/100 g were exclusively detected in berries from Area 1. The 10-formylfolate and folic acid
297 content in sea buckthorn berries from both growing areas was below the limit of detection (<
298 0.6 µg/100 g). Comparing our results with a previous study on folates in sea buckthorn berries
299 [15] we found our data in good accordance as the authors reported a total folate concentration
300 in sea buckthorn berries of about 39 µg/100 g and 5-methyltetrohydrofolate was characterized
301 as the predominating folate vitamer in berries. Traces of tetrahydrofolate were observed but
302 were far below the detection limit [15]. In consideration of cultivar variation the 5-
303 methyltetrahydrofolate concentration of sea buckthorn berries in the study of Strålsjö et al.
304 [15] corresponded to our measured amount of about 31 µg/100 g (Area 2) and 68 µg/100 g
305 (Area 1), respectively. Our investigations on folate derivatives in sea buckthorn berries and
306 related products with total folate content of 36 µg/100 g (Area 2) and 81 µg/100 g (Area 1)
307 showed that berries of *Hippophaë rhamnoides* L. ssp. *rhamnoides* pertain to a particular
308 species of fruits containing high concentrations of folate [15, 16]. When comparing the folate
309 content of sea buckthorn berries with that of other berries, the berries from Area 1 containing
310 81 µg/100g were nearly as high in folates as rose hips, which showed the highest folate
311 content of 96 µg/100g among all berries under study [15].
312 The Recommended Dietary Allowance for women and men is set to 400 µg/day of dietary
313 folate equivalents. Fruits and berries are one of the main folate sources and may provide up to
314 15% of the daily folate intake [15]. Assuming that the average serving size of *Hippophaë*
315 juice in mixtures of fruit juices causing the characteristic sour taste does not exceed 100 g, the

316 folate contribution of sea buckthorn juice (Area 1) may represent a ”excellent source”
317 exceeding 20% for the achievement of the recommended dietary intake for all adults
318 according to the definitions of the U.S. Food and Drug Administration [30].
319
320 Processing effects on folate vitamers in juice and concentrate
321
322 The aim of our current study was to evaluate the stability of folate vitamers in sea buckthorn
323 juice and juice concentrate (Area 2) under the influence of processing. After preheating and
324 mashing, a decanter machine was used for the gentle separation into juice and pomace
325 without application of high pressure. Furthermore, commercial sea buckthorn juice production
326 includes a high-temperature-short-time process before aseptic filling. The folate vitamers
327 differ in their characteristics concerning oxidative degradation, thermal stability, and the pH
328 dependence of their stability. In contrast to this, the length of the glutamyl side chain had
329 marginal or no influence on the stability properties of the folate derivatives [31].
330 Due to the separating and heating process a degradation of folate vitamers in sea buckthorn
331 juice and juice concentrate had to be expected.
332 *Tetrahydrofolate*
333 The juice production process entailed a total degradation of the tetrahydrofolate in sea
334 buckthorn juice (Area 2) (Fig. 3). Particularly tetrahydrofolate is extremely sensitive to
335 physical and chemical factors such as temperature, oxygen and pH values [31-33]. Pearson
336 [32] described the oxidation of tetrahydrofolate as a free radical chain process. The
337 degradation of tetrahydrofolate under acidic conditions (pH 4) corresponded approximately to
338 the rate of formation of the major product *p*-aminobenzoylglutamic acid. Pterin was the major
339 pteridine product which resulted from the complete oxidative degradation of 7,8-
340 dihydropterin. Only a small amount of 6-formylpterin was formed at pH 4 (Fig. 4). The
341 described reaction products of decomposition are biologically inactive [33], and, therefore,

were not analyzed in the present study. The degradation of the tetrahydrofolate content in sea buckthorn juice (Area 2) was obviously caused by the homogenous distribution of tetrahydrofolate in a liquid matrix and a pH value of 2.8 for sea buckthorn juice compared to other juice matrices. Butz et al. [25] investigated the influence of high-pressure treatment at 25 °C and 80 °C on folates in orange juice and model media. The experimental data for the model orange juice are well in line with our results. Upon a treatment at 600 MPa and 80 °C tetrahydrofolate was approximately completely degraded after 6 minutes at a pH value of 3.5, without the application of pressure a decay of about 60% after 6 minutes was observed. Freshly squeezed orange juice appeared more stable than the model juice. In that case a lower degradation of the most sensitive folate vitamer tetrahydrofolate with losses up to 20% was detected.

5-Methyltetrahydrofolate

The content of 5-methyltetrahydrofolate (31 µg/100 g) was approximately unchanged during the whole processing from the berries to the concentrate (Fig. 3). Pearson [32] described that the oxidation rate of 5-methyltetrahydrofolate vitamer is lower in comparison to the non-methylated tetrahydrofolate derivative. Moreover, the author [32] postulated that the reaction rate of oxidation is dependent on steric factors. According to his hypothesis, the oxygen molecule must approach the C4, C4a, N5 region of the folate vitamer (Fig. 1) in close spatial proximity. A methyl group at N5 would decrease the number of effective collisions due to the existing steric shielding [32].

Furthermore, the results of our study were in good accordance with the investigations of Butz et al. [25]. 5-Methyltetrahydrofolate was slightly decreased in model orange juice by about 10-30% upon a treatment at 25 °C and 80 °C (no use of pressure or 600 MPa) up to 24 minutes. During thermovacuum evaporation process (five stage evaporator, 80–85 °C) leading from juice to concentrate, the pH of the final product was reduced to the value of 2.6. Interestingly, our results revealed that 5-methyltetrahydrofolate content in juice concentrate

was only slightly decreased resulting in a loss of 10%. This is in contrast to the results of Vahteristo et al. [16], who reported that a juice concentrate prepared from a selection of berries and fruits had a folate content below the detection limit. Obviously, the concentration process during thermovacuum evaporation used in our study (five stage evaporator, 80–85 °C) occurs moderately. Regrettably, data from the study by Vahteristo et al. [16] contain no details about the technical parameters in the used concentrate production process.

5-Formyltetrahydrofolate

The investigated sea buckthorn berries (Area 2) exhibited trace amounts (1 μg/100 g) of 5-formyltetrahydrofolate, which were completely lost during the juice production (Fig. 3). Likewise 5-methyltetrahydrofolate, the 5-formyltetrahydrofolate derivative is relatively stable to air oxidation. However, this vitamer is susceptible to low pH values (pH 1.0-2.0), in particular at elevated temperatures, which results in the formation of 5,10-methenyltetrahydrofolate by the cleavage of a water molecule [25, 34]. Due to the low content of 5-formyltetrahydrofolate in the berries, the latter degradation product was neither detectable in juice nor in the concentrate.

The production process of sea buckthorn juice and juice concentrate (Area 2) resulted in a degradation of the total folate vitamers contents of 19% and 25%, respectively. In summary it can be ascertained that the processing of sea buckthorn juice and juice concentrate (Area 2) resulted in a lower degradation than expected.

Storage stability in food systems

To investigate the influence of temperature on folate vitamers in *Hippophaë* juice (Area 1) storage experiments were performed under two consumer storage conditions (6 °C, 25° C), and also under accelerated aging conditions (40° C) for seven days. To study the effect of temperature on contents of the folate derivatives, stored juices were analyzed after 72 h and

thereafter in intervals of 48 h. Due to the results of the processing effects the storage investigations were focussed on the stability of the 5-methyltetrahydrofolate derivative. Fig. 5 shows the effect of storage on the folate content in sea buckthorn juice at 6 °C, 25 °C, and at 40 °C over a period of 7 days. The contents of 5-methyltetrahydrofolate and of the total folate were approximately unchanged during the storage at 6 °C after seven days (Fig. 5). Almost identical degradation of 5-methyltetrahydrofolate in the range of about 17-20% at 25 °C and 40 °C, respectively, were observed for juices after seven days of storage. Interestingly, in comparison to these results the total folate content decreased after seven days at 25 °C slowly by 5% and the degradation increased at 40 °C up to 17% after seven days. The 17% decline of 5-methyltetrahydrofolate at 25 °C and the almost unaffected measured content of total folate under the same storage conditions can be explained by an increase in the 5-formyltetrahydrofolate content of 12 µg/100 g, which compensates the loss of 5-methyltetrahydrofolate. In the same manner this phenomenon was observed after 72 hours at 40 °C. Within the next 48 hours the reaction was reversed (Fig. 5). The formation of 5-formyltetrahydrofolate may result from an enzymatic conversion of 5-methyltetrahydrofolate in the course of the usual folate metabolism in plant tissue. Thus, merely in accelerated storage experiments a loss of 5-methyltetrahydrofolate and consequently of the total folate content was affected by storage time and temperature. After seven days of storage at 40 °C the 5-methyltetrahydrofolate amount and the total folate content decreased by 17%. This is well in line with the results of Jastrebova et al. [26], who observed during storage of pickled beetroots for 3 and 15 months at ambient temperature a degradation of 5-methyltetrahydrofolate of 6-20% and 22-28%, respectively. This confirmed the moderate loss of folate during consumer storage temperature conditions. Further studies investigated the heating effects on folate content at different temperatures (up to 100 °C) and time ranges in model systems [35-38]. Analysis of kinetic data suggested that the degradation is following a first-order model. Mnkeni and Beveridge [35] investigated the decomposition of 5-

methyltetrahydrofolate in apple juice (pH 3.4, 50-70 °C) and in tomato juice (pH 4.3, 100-130 °C). 5-Methyltetrahydrofolate degradation was consistent with a first order kinetic model. The rates of depletion of 5-methyltetrahydrofolate in apple juice and tomato juice were higher than in buffer systems. Since in our study the 5-methyltetrahydrofolate content was only affected at 40 °C a calculation of kinetic data was not accomplishable in contrast to our investigations of pantothenic acid in sea buckthorn juice [28]. Lucock et al. [36] determined the influence of temperature and of the pH value on the stability of 5-methyltetrahydrofolate. The rates of degradation increased with temperature. At higher pH values (pH 9.0) the rates of decomposition are much faster compared to low pH values (pH 3.5). One of the characteristics of sea buckthorn berries are low pH-values of approximately 2.8 in comparison to other berry fruits. This may contribute to the increased stability of 5-methyltetrahydrofolate. Moreover, the latter vitamer is more stable in the presence of antioxidants such as ascorbate [38]. This protective effect is due to the reduction of the dissolved oxygen concentration since ascorbate acts as an oxygen scavenger [39]. In prior investigations of our laboratory about 400 mg ascorbic acid /100 g juices was found (non published data), which provides stability of the main folate vitamer 5-methyltetrahydrofolate. Another cause for the constant level of 5-methyltetrahydrofolate might be its formation from other vitamers such as tetrahydrofolate by reaction with formaldehyde originating from ascorbic acid degradation [40].

Results of our study clearly demonstrated that temperature effects - the most important consumer storage parameter of 6 °C and 25 °C - do not influence the total folate content of sea buckthorn juice within seven days storage.

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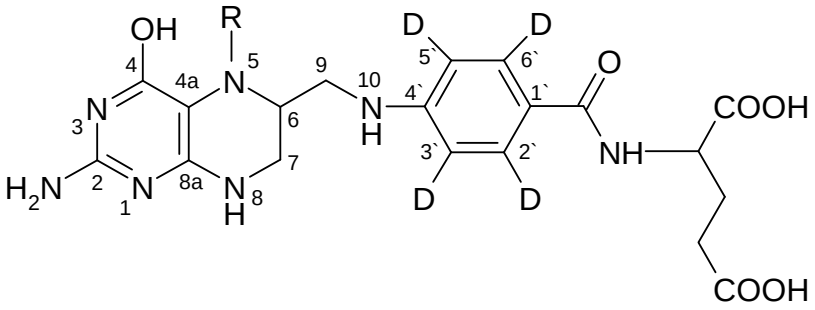
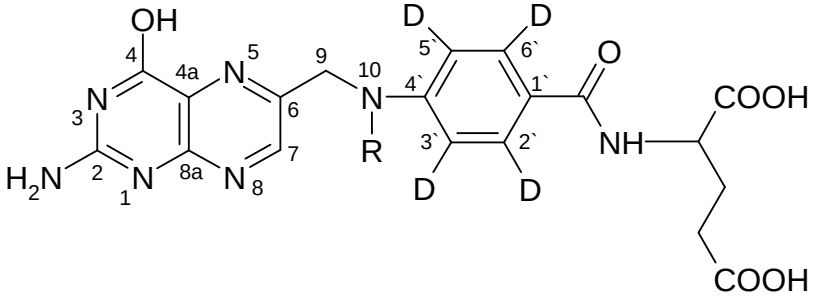


[$^2H_4$]tetrahydrofolate R = H

[$^2H_4$]5-methyltetrahydrofolate R = CH_3

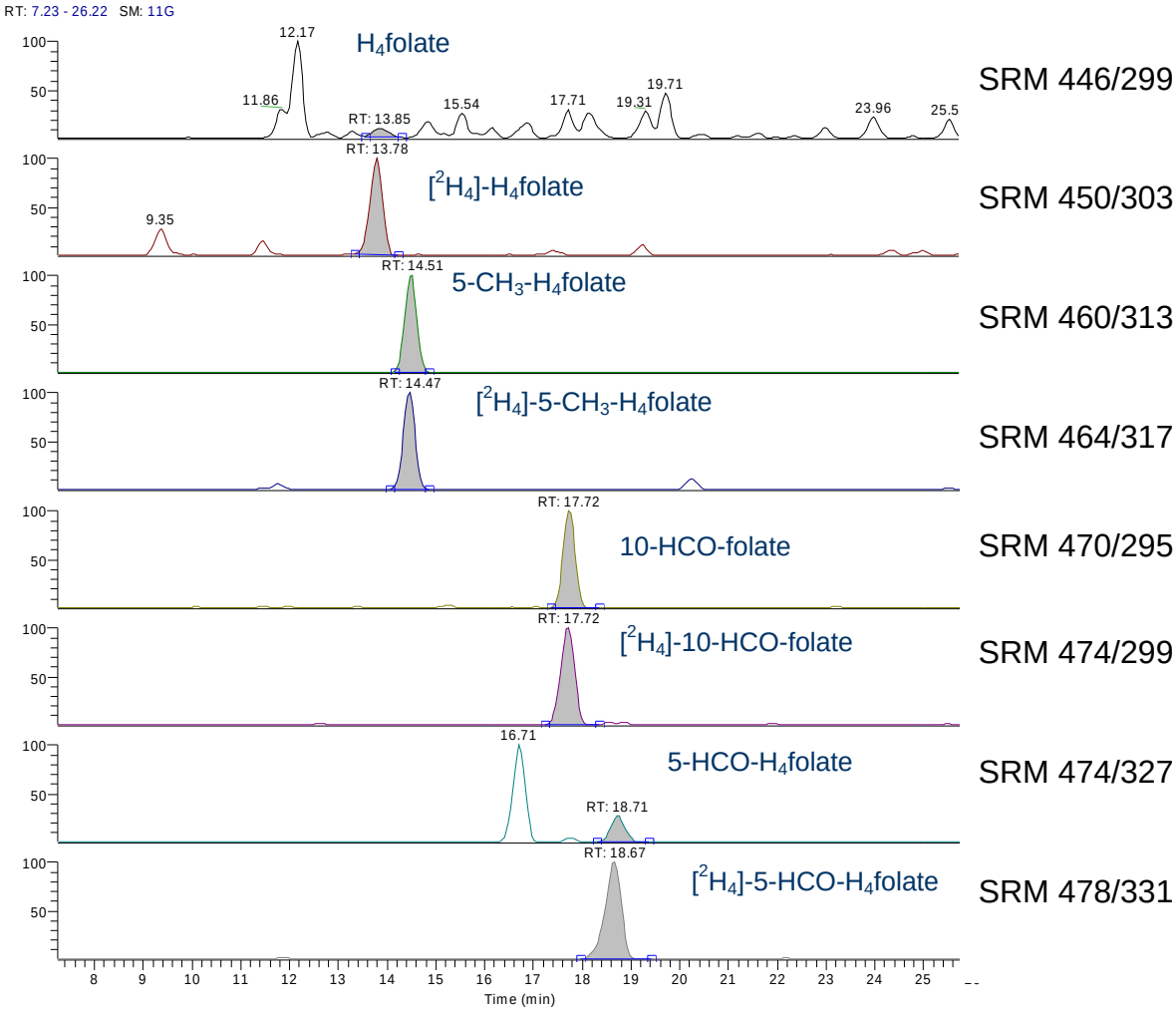
[$^2H_4$]5-formyltetrahydrofolate R = CHO



[$^2H_4$]folic acid R = H

[$^2H_4$]10-formylfolate R = CHO

Fig. 1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H4folate
SRM 446/299
[2H4]-H4folate
SRM 450/303
5-CH3-H4folate
SRM 460/313
[2H4]-5-CH3-H4folate
SRM 464/317
10-HCO-folate
SRM 470/295
[2H4]-10-HCO-folate
SRM 474/299
5-HCO-H4folate
SRM 474/327
[2H4]-5-HCO-H4folate
SRM 478/331
Time (min)

Fig. 2

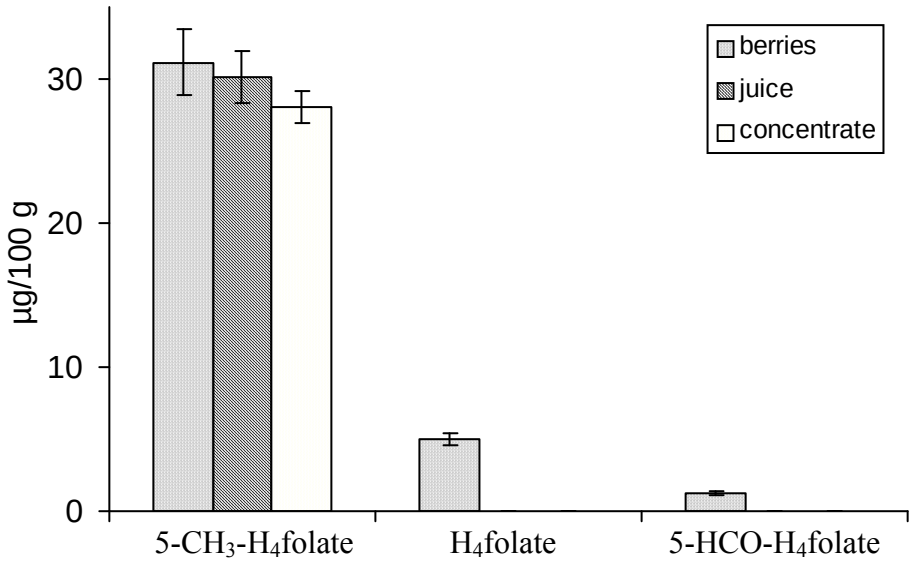
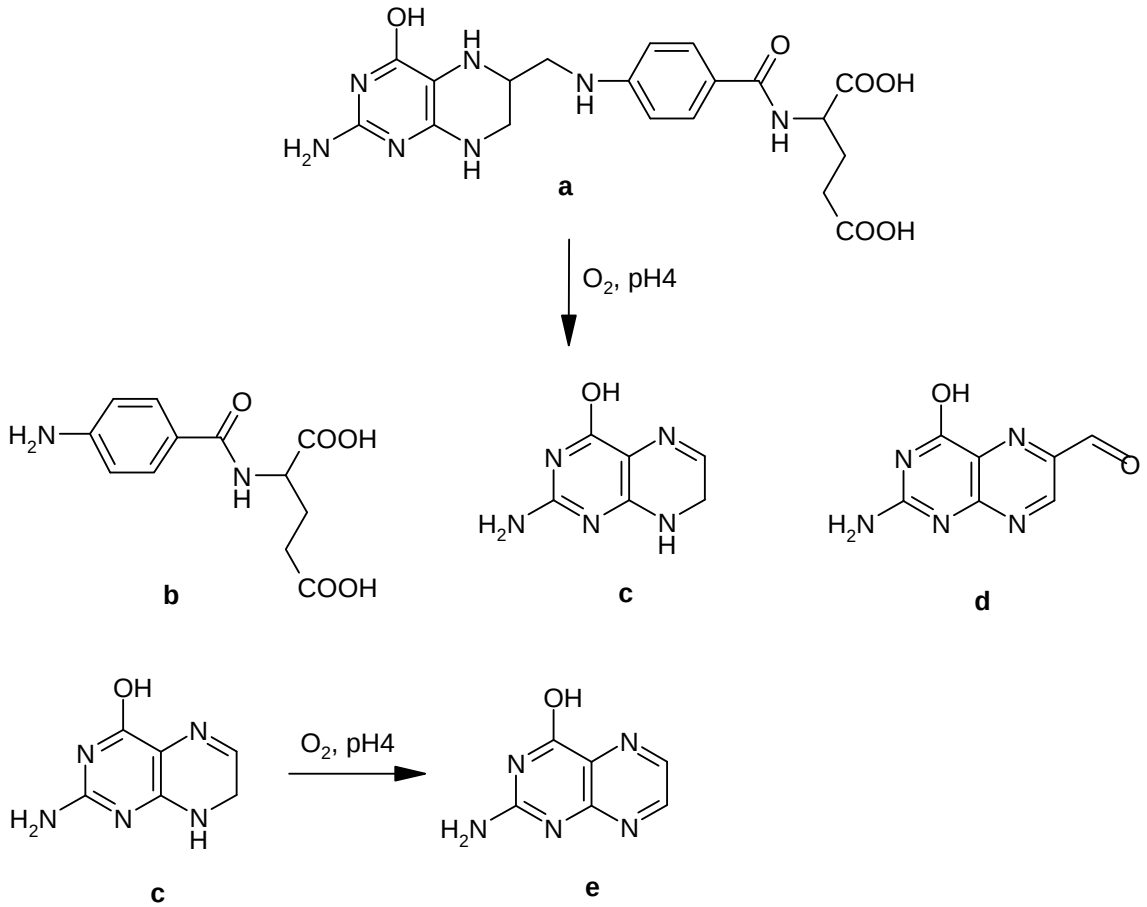
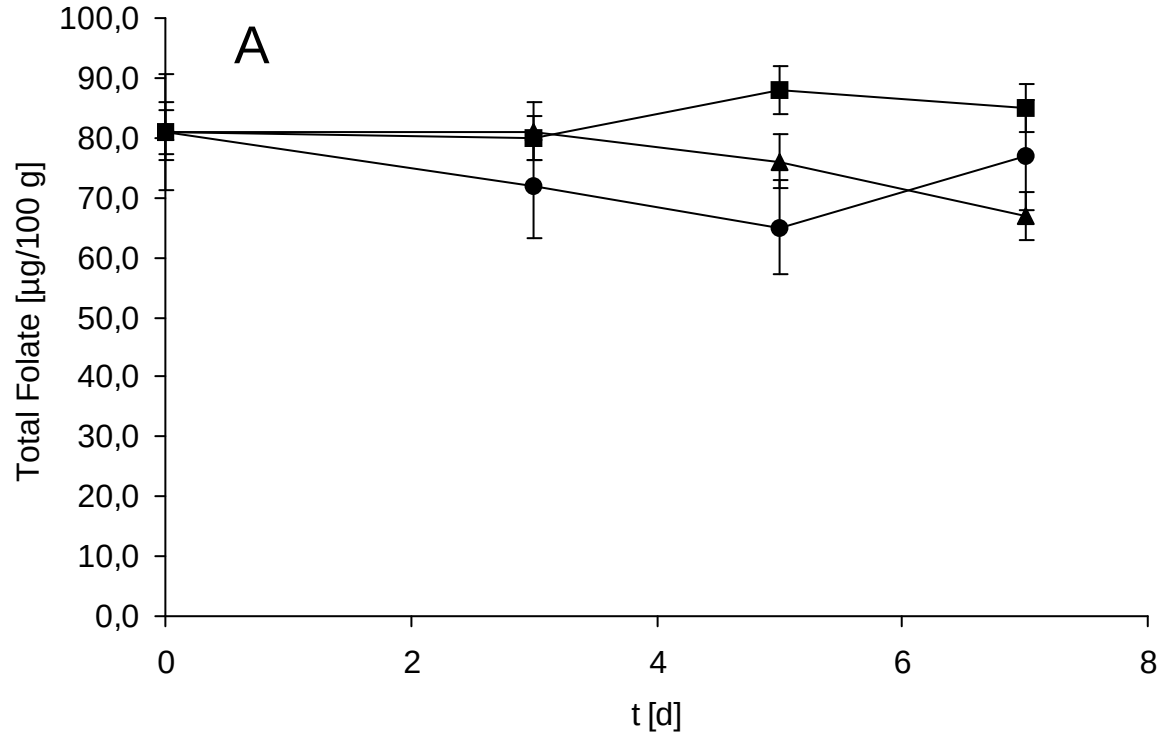

berries
juice
concentrate
µg/100 g
30
20
10
0
5-CH3-H4folate
H4folate
5-HCO-H4folate

Fig. 3


OH
H
N
N
N
H
H2N
N
H
O
COOH
N
H
COOH
a
O2, pH4
H2N
O
COOH
N
H
COOH
b
OH
N
N
H2N
N
N
H
c
OH
N
N
H2N
N
N
O
d
OH
N
N
H2N
N
N
H
c
O2, pH4
OH
N
N
H2N
N
N
e

496

497 **Fig. 4**



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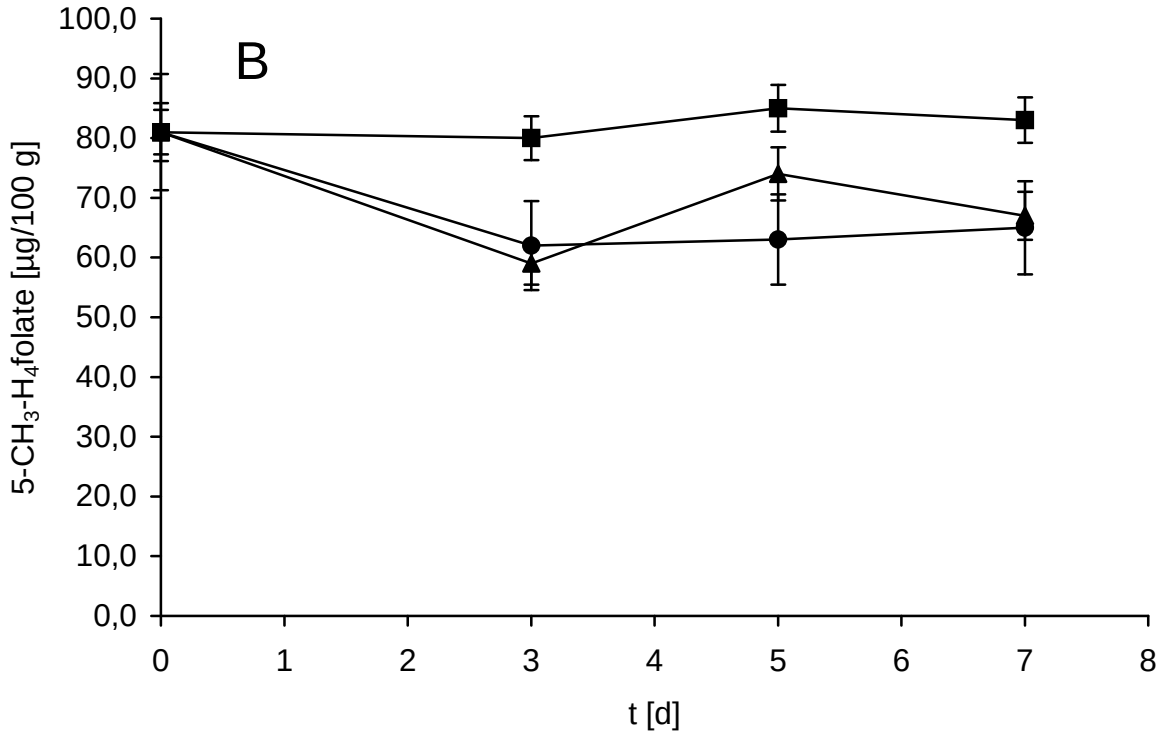

499

500 **Fig. 5 A and B**

501

502

Legends to the figures.

Figure 1 Structures of the folate vitamers applied to stable isotope dilution assay.

Figure 2 LC-ESI-MS/MS chromatogram in positive ionization mode of a folate extract of sea buckthorn juice. Selected reaction monitoring (SRM) traces of folate vitamers and their isotopologues: *m/*z precursor ion/*m/*z product ion

Figure 3 Decrease of 5-methyltetrahydrofolate (5-CH_3-H_4folate), tetrahydrofolate (H_4folate) and 5-formyltetrahydrofolate (5-HCO-H_4folate) of sea buckthorn berries (Area 2) subjected to the commercial manufacturing technique for production of juice and juice concentrate (cf. Materials and Methods)

Figure 4 Acidic autoxidation of tetrahydrofolate: **a**: tetrahydrofolate, **b**: *p*-aminobenzoylglutamic acid, **c**: 7,8-dihydropterin, **d**: 6-formylpterin, **e**: pterin.

Figure 5 Effects of storage for 7 days on **A:** total folate content and **B:** 5-methyltetrahydrofolate degradation in sea buckthorn juice of Area 1 at cold storage (6 °C, 7 days) (■), room temperature (25 °C, 7 days) (●), and elevated temperature (40 °C, 7 days) (▲).

529 **References**

530 1. Guliyev VB, Gul M, Yildirim A (2004) J Chromatogr B 812:291-307

531 2. European Directorate For The Quality of Medicines and Health Care (2005) Acidum
532 folicum. In: European Pharmacopoeia, 5th ed, The Council of Europe, Govi, Eschborn,
533 Germany

534 3. Moss GP (1986) Nomenclature and symbols for folic acid and related compounds.
535 International Union of Pure and Applied Chemistry and International Union of Biochemistry
536 and Molecular Biology, IUPAC-IUB Joint Commission on Biochemical Nomenclature
537 (JCBN), world wide web version, http://www.chem.qmul.ac.uk/iupac/misc/folic.html

538 4. Ball GFM (2006) Vitamins in foods. Analysis, bioavailability, and stability. CRC Press
539 Taylor & Francis Group, Boca Raton

540 5. Wagner C (1996) J Nutr 126:1228S-1234S

541 6. Wald N, Sneddon J, Densem J, Frost C, Stone R (1991) Lancet 338:131-137

542 7. Snowdon DA, Tully CL, Smith CD, Riley KP, Markesbery WR (2000) Am J Clin Nutr
543 71:993-998

544 8. Regland B (2005) Prog Neuro-Psychopharmacol Biol Psych 29:1124-1132

545 9. Robinson K (2000) Heart 83:127-130

546 10. Lindenbaum J, Nath BJ (1980) Br J Haematol 44:511-513

547 11. Zhang S, Hunter DJ, Hankinson SE, Giovannucci EL, Rosner BA, Colditz GA, Speizer
548 FE, Willett WC (1999) J Am Med Assoc 281:1632-1637

549 12. Kim YI (2003) J Nutr 133:3731S-3739S

550 13. Rezk BM, Haenen GRMM, van der Vijgh WJF, Bast A (2003) FEBS Lett 555:601-605

551 14. National Academy of Sciences, Institute of Medicine, Food and Nutrition Board (1998)
552 Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12,
553 pantothenic acid, biotin, and choline. National Academy Press, Washington, DC

554 15. Strålsjö L, Åhlin H, Witthöft CM, Jastrebova J (2003) Eur Food Res Technol 216:264-269

555 16. Vahteristo L, Lehikoinen K, Ollilainen V, Varo P (1997) Food Chem 59:589-597
556 17. Tamura T, Mizuno Y, Johnston KE, Jacob RA (1997) J Agric Food Chem 45:135-139
557 18. Shrestha AK, Arcot J, Paterson J (2000) Food Chem 71:545-552
558 19. O´Broin JD, Temperley IJ, Brown JP, Scott JM (1975) Am J Clin Nutr 28:438-444
559 20. Pawlosky RJ, Flanagan VP (2001) J Agric Food Chem 49:1282-1286
560 21. Freisleben A, Schieberle P, Rychlik M (2002) J Agric Food Chem 50:4760-4768
561 22. Freisleben A, Schieberle P, Rychlik M (2003) Anal Bioanal Chem 376:149-156
562 23. Rychlik M (2004) J Food Comp Anal 17:475-483
563 24. Rychlik M, Englert K, Kapfer S, Kirchhoff E (2007) J Food Comp Anal 20:411-419
564 25. Butz P, Serfert Y, Fernandez Garcia A, Dietrich S, Lindauer R, Bognar A, Tauscher B
565 (2004) J Food Sci 69**:**SNQ117-SNQ121
566 26. Jastrebova J, Witthöft C, Grahn A, Svensson U, Jägerstad M (2003) Food Chem 80:579-
567 588
568 27. Beveridge T, Harrison JE, Drover J (2002) J Agric Food Chem 50:113-116
569 28. Gutzeit D, Klaubert B, Rychlik M, Winterhalter P, Jerz G (2007) J Agric Food Chem
570 55:3978-3984
571 29. Gutzeit D, Baleanu G, Winterhalter P, Jerz G (2007) J Food Sci 72:C491-C497
572 30. U.S. Food and Drug Administration (1999) Food Labeling Guide-Appendix B, online
573 published: http://www.cfsan.fda.gov/~dms/flg-6b.html
574 31. Gregory JF III (1985) Chemical changes in vitamins during food processing. In:
575 Richardson T, Finley JW (eds) Chemical changes of food during processing. Van Nostrand
576 Co., New York
577 32. Pearson AJ (1974) Chem Ind 6:233-239
578 33. Reed LS, Archer MC (1980) J Agric Food Chem 28:801-805
579 34. Cosulich DB, Roth B, Smith Jr JM, Hultquist ME, Parker RP (1952) J Am Chem Soc
580 74:3252-3263

581 35. Mnkeni AP, Beveridge T (1983) J Food Sci 48:595-599

582 36. Lucock MD, Green M, Hartley R, Levene MI (1993) Food Chem 47:79-86

583 37. Nguyen MT, Indrawati, Hendickx M (2003) J Agric Food Chem 51:3352-3357

584 38. Oey I, Verlinde P, Hendrickx M, Van Loey A (2006) Eur Food Res Technol 223:71-77

585 39. Hawkes JG, Villota R (1989) Crit Rev Food Sci Nutr 28:439-538

586 40. Wilson SD, Horne DW (1983) Proc Nat Acad Sci 80:6500-6504

587