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Picomolar quantitation of free sulfite in foods by means of [^{57}Co]hydroxocobalamin and radiometric chromatography of [^{57}Co]sulfitocobalamin
Method, applications and significance of coexisting sulfides

Robert A. Beck[a,b,*], Jean M. Anes[a,b], Lisa M. Savini[a,b], Robert A. Mateer[a,b]

[a]*Framingham Institute for Food Science, Technology and Nutritional Biochemistry, Framingham State College, 100 State Street, Framingham Center, Framingham, MA 01701, USA*

[b]*Department of Chemistry and Food Science, Framingham State College, 100 State Street, Framingham Center, Framingham, MA 01701, USA*

Abstract

The concentration dependent reaction of sulfite with ^{57}Co-labeled hydroxocobalamin (OH^{57}CoCbl) to produce a sulfitocobalamin (SO_3^{57}CoCbl) adduct served as a quantification strategy for foodborne sulfite residues freely extracted into pH 5.2, 0.05 *M* acetate buffer. SO_3^{57}CoCbl was then resolved using SP-Sephadex C-25 gel chromatography and its radiometric detection allowed calculation of a standard logit plot from which unknown sulfite concentrations could be determined. The sulfite detection range was 6.0 n*M*–0.3 p*M* with respective relative standard deviations of 4.4–29.4% for 50-μl samples. Individual incidences of foodborne sulfite intolerances provoked by L-cysteine or sulfite additive use in bakery products, which remained undetected using conventional sulfite analytical methods, underscored the quantitative value of the method. The analytical significance and occurrences of detectable sulfides coexisting with foodborne sulfite residues was also addressed

Keywords: Food analysis; Sulfite; Sulfides; Hydroxocobalamin; Cysteine; Vitamins

1. Introduction

Sulfitocobalamin (SO_3Cbl) is one of several identified cobalamin adducts [1–4], but it has uncertain significance and origins in biological materials [5–9]. Although natural routes producing SO_3Cbl are poorly understood, in vitro exposure of hydroxocobalamin (OHCbl or vitamin B_{12a}) to free sulfite or sulfur dioxide readily produces SO_3Cbl. The formation constant for sulfite reaction with OHCbl is second only to the reaction of cyanide with OHCbl to produce cyanocobalamin (CNCbl or vitamin B_{12}) [3].

During previous studies that explored the ability of dietary sulfite to alter SO_3Cbl occurrences in brain tissue [10], it was clear that the strong formation constant for sulfite reaction with OHCbl could serve as a basis for free sulfite quantification [11]. Using principles of radiometric saturation analysis [12], uniformly fixed amounts of high specific activity [^{57}Co]hydroxocobalamin (OH^{57}CoCbl) (e.g., 40 000 cpm) were individually incubated with a series of

*Corresponding author. Framingham Institute for Food Science, Technology and Nutritional Biochemistry, 100 State Street, Hemenway Hall, FSC, Framingham Center, Framingham, MA 01701, USA. Tel.: +1-508-6264-755; fax: +1-508-6264-718.

E-mail address: beckra@worldnet.att.net (R.A. Beck)

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standard 0.3 p*M*–6.0 n*M* sulfite concentrations present in 50-μl sample volumes. Consequently, the reaction of OH^{57}CoCbl with any available sulfite produced corresponding increases in detectable amounts of SO_3^{57}CoCbl. The specific detection of SO_3^{57}CoCbl, formed at each sulfite concentration, was verified by its discriminative gel chromatographic separation using a SP-Sephadex C-25 column [9,10,13,14]. Radiometric counts of SO_3^{57}CoCbl produced at each respective sulfite concentration, provided data for calculating a standard analytical curve from which unknown amounts of sulfite could be determined.

The standard curve was constructed as a conventional logit plot [12] where each abscissa value was scaled as the $\log_{10}$ of a standard molar sulfite concentration. Ordinate data for each standard sulfite concentration was expressed as a corresponding logit-based radiometric binding value for SO_3^{57}CoCbl formed from OH^{57}CoCbl. Thus, any unknown sample sulfite concentration was determined from the standard logit plot, after sample incubation with OH^{57}CoCbl (e.g., 40 000 cpm) and radiometric calculation of its SO_3^{57}CoCbl formation as a logit-based value.

Realistic advantages of this radiochromatographic analysis (RCA) for free sulfite were revealed in a series of incidences, where dietary sulfite intolerances of seven individuals correlated with sulfite residues in bakery products. Analysis of the baked products using conventional methods failed to confirm any detectable sulfite. These methods included the Monier–William's method [15] and ion-exclusion chromatography with electrochemical detection (ED) [16,17] which, respectively, permitted total sulfite detection limits of 10 ppm and ≥0.1 ppm. The high sensitivity and discrimination of the RCA method reported however, detected evidence of free sulfite in all baked products and it offered sulfite sensitivity as low as 0.3 p*M* (24 pg) in a sample volume of only 50 μl.

The term sulfite as used in this paper is an all inclusive term for every form of sulfur in the 4+ oxidation state that occurs in foods, pharmaceuticals and cosmeceuticals. These forms typically include sulfur dioxide (SO_2), metabisulfite ($S_2O_5^{2-}$), hydrogensulfite (HSO_3^-) and sulfite (SO_3^{2-}). Sulfites added to foods serve as antioxidants, antimicrobials and dough conditioners where they undergo a variety of reactions [18]. Depending on the analytical procedure, sulfites can be described as free, reversibly or irreversibly bound. Free sulfites are often defined as those forms where acidification readily converts sulfite into sulfur dioxide which can be quantitatively analyzed upon distillation. Reversibly bound sulfites typically yield an equivalent of sulfur dioxide for analysis only after heat and acid or alkali treatments [16,19] are applied. These forms are modeled as carbonyl addition products exemplified by $=C(OH)SO_3^-$. Sulfites that bind to food matrices and fail to release an equivalent of sulfur dioxide during protracted heating and acidic conditions are considered irreversibly bound. Thiosulfonates ($R\text{-}S\text{-}SO_3^-$) typify this model. Regulatory compliance guidelines for sulfite applications are often based on the combined total of free, reversible and irreversible forms. Free sulfite in this study specifically refers to sulfite in food or biological material which is readily extracted into mild pH 5.2, 0.05 *M* acetate buffer without severe acidification. The extracted sulfite then undergoes adduct formation with OH^{57}CoCbl which can be chromatographically resolved and radiometrically quantitated.

The object of this study was to develop a radiometric quantification method for sulfite, uniquely dependent on its reaction with OH^{57}CoCbl, which was capable of detecting picogram (pg) amounts of sulfite in small sample volumes (e.g., ≤50 μl). The application strategy was geared to meet those sensitive and selective verification challenges where sulfite may exert a clinical, subclinical or nutritional impact on hyperintolerant individuals. The method not only allowed low sulfite detection limits of ≥0.3 p*M*/50 μl of sample, but it collaterally detected the presence of sulfides that often coexisted with foodborne sulfite residues. Although sulfides could be detected, these were not a subject of quantification in this report. Nevertheless, sulfide detection in an analytical framework designed for sulfite quantification, also permitted source differentiation of sulfur dioxide evolved from the use of L-cysteine in baked products as opposed to the direct use of sulfite additives in food formulations.

2. Experimental

Quantification of free sulfites relied on five uni-

formly implemented steps including: (1) the construction of a cation-exchange gel chromatographic column; (2) preparation of radiochromatographic SO_3^{57}CoCbl standards and calculation of an analytical logit plot from which unknown sample sulfite concentrations were determined; (3) sample preparation for sulfite analysis; and (4) an assessment of radiotracer recovery used for probing sulfite levels in food samples. Beyond these normal application requirements, this study required an added step (5) to characterize the significance of RCA elution profiles shown by some food samples. This involved preparing volatile and nonvolatile reactants to give RCA identifiable OH^{57}CoCbl adducts indicative of indirect food sulfite residue origins in foods. Without this step, any detectable sulfite residue produced from the thermal decomposition of L-cysteine could be confused with direct use of inorganic sulfite since both additives are used in bakery and food products.

2.1. Gel column preparation

Preparation of the SP-Sephadex C-25 cation-exchange gel (Pharmacia, Piscataway, NJ, USA) was consistent with earlier reported methods for the construction of a 10.0×1.5 cm gel column [10]. Deaerated, sulfur dioxide-free, high-purity water (Burdick and Jackson, Muskegon, MI, USA) was supplied to the column as a mobile phase at 125 μl/min. All polypropylene tubing serving the column, its sample injector and the column eluent flows was 0.5 mm I.D. Standard column elution fractions ensuring isolation of SO_3Cbl and all other cobalamin adducts were fixed at 160 μl.

2.2. Preparation of standard SO_3^{57}CoCbl concentrations and logit plot determination

Radioactive SO_3^{57}CoCbl calibration standards were prepared for construction of an analytical curve against which unknown free sulfite concentration levels could be determined. Preparation of these standards relied on the initial conversion of high specific activity CN^{57}CoCbl (389 kBq) (ICN Pharmaceuticals, Costa Mesa, CA, USA) to OH^{57}CoCbl. The CN^{57}CoCbl (0.05 μg/ml) was photolytically transformed at 5.0°C into OH^{57}CoCbl by addition of 25 μl of 2.0 m*M* HCl followed by illumination for 24 h with a 500 W lamp. This was subsequently diluted with 0.05 *M* sodium acetate (pH 5.2) to give 40 000 cpm/100 μl. Radioactive SO_3^{57}CoCbl standards were routinely prepared, one at a time, by incubating 100 μl of OH^{57}CoCbl for 30 min with sulfite concentrations ranging from 0.3 p*M*–6.0 n*M* in 50 μl of acetate buffer. Standards were prepared only in 45×11 mm polypropylene tubes. The resulting 150-μl volume of OH^{57}CoCbl, reacted with sulfite anions, was then introduced onto the SP-Sephadex C-25 column for SO_3^{57}CoCbl detection. Each 160-μl fraction eluted from the column was radiometrically monitored using a Clinigamma Twin System (Wallac LKB, Gaithersburg, MD, USA). Radiometric counts per minute (cpm) resulting from elution of standard concentration levels, provided data for calculating a standard logit-based analytical curve [12] from which unknown sulfite concentrations could be determined. SO_3^{57}CoCbl demonstrated peak elution at 8000–8160 μl consistent with previously reported results [10].

Logit-based values were calculated using the formula $\ln [(B/B_0)/(1-B/B_0)]$. The "B_0" term represented the total fixed amount of OH^{57}CoCbl (e.g., 40 000 cpm) available for reaction with sulfite to give SO_3^{57}CoCbl; and, "*B*" corresponded to the respective cpm data for the reaction of individual sulfite standards from 0.3 p*M*–6.0 n*M*, with an identical fixed level of OH^{57}CoCbl, to give sulfite-dependent concentrations of SO_3^{57}CoCbl. Thus, logit values (*y*-axis) were plotted for radiometrically quantifiable SO_3^{57}CoCbl occurrences versus the $\log_{10}$ of molar sulfite concentrations (*x*-axis) present in 50-μl samples. This approach gave a standard curve against which logit values for similarly prepared unknown amounts of sulfite could be quantified. All reactions involving free sulfite with OH^{57}CoCbl adhered to a 30 min incubation time at 25°C.

2.3. Preparation of solid or aqueous samples for sulfite quantification

Samples suspected of containing free sulfites were, respectively, diluted and/or blended with 0.05 *M* sodium acetate buffer at pH 5.2 in the range of a 0.5–0.001 ratio (w/w) as necessary. All diluted aqueous samples or extracts of solid samples were clarified by centrifugation at 5000 *g* followed by

0.22 μm filtration [20]. A 50-μl sample filtrate volume, typically containing 0.3 p*M*–6.0 n*M* of free sulfite, was allowed to react with 100 μl of OH^{57}CoCbl (40 000 cpm) for 30 min consistent with standard SO_3^{57}CoCbl preparations specified above. After this reaction period, the 150-μl volume was administered to a SP-Sephadex C-25 column. The radiometric resolution of SO_3^{57}CoCbl served as the basis for calculating a corresponding logit value, and the amount of free sulfite in any sample was determined from the standard logit plot.

2.4. OH^{57}CoCbl radiotracer recovery from samples and monitoring for reformation of CN^{57}CoCbl

The quantitative validity of the sulfite radioassay demanded unimpeded kinetic binding interaction between a fixed amount of available OH^{57}CoCbl and variable amounts of free sulfite. Thus, inadvertent binding of OH^{57}CoCbl to unknown compounds modeled by R-proteins [21–23] in 0.22 μm filtered samples could corrupt the assay. This problem could appear in two ways. First, a loss of quantitative linear radiometric assay response may exist over the course of serial sulfite-containing sample dilutions. A second scenario signaling analytical trouble may appear as a gross inequity between the initial radioactivity level of the OH^{57}CoCbl probe, and the detectability of SO_3^{57}CoCbl if sample sulfite concentrations largely exceed those required to convert all OH^{57}CoCbl into SO_3^{57}CoCbl.

For this study, sample-specific radiotracer recovery was assessed by adding sulfite to filtered samples so their 50-μl volume contained $\geq 4.2 \cdot 10^{-8}$ *M* sulfite prior to the 30 min incubation period with OH^{57}CoCbl (e.g., 40 000 cpm). This maximized the conversion of available OH^{57}CoCbl into SO_3^{57}CoCbl whose formation could be validated using RCA. Recoveries of initial OH^{57}CoCbl levels, detected as SO_3^{57}CoCbl using the specified conditions, were typically 74.9±2.3% for triplicate assays. This recovery level was interpreted as confirmation that disruptive binding interactions between the radiotracer and unknown sample constituents failed to occur.

Verification that any of the OH^{57}CoCbl radiotracer stock solution did not undergo reformation to give CN^{57}CoCbl, before its analytical use for sulfite detection, was ensured by routine monitoring of the tracer on a four day cycle. This was accomplished by radiochromatography of a 100-μl volume of the tracer using the column chromatographic procedure already described.

2.5. Volatile and nonvolatile reactant preparations for RCA OH^{57}CoCbl adduct studies

In some cases, it was necessary to establish standard radiochromatographic elution profiles for cobalamin adducts when volatile sulfur compounds reacted with OH^{57}CoCbl. Sulfur compounds were of special interest, particularly those released during the thermal decomposition of L-cysteine (Sigma, St. Louis, MO, USA) as well as sulfur volatiles naturally formed over sulfur dust.

OH^{57}CoCbl reactive volatiles originating from L-cysteine were prepared in individual 10.0×1.0 cm reaction tubes each equipped with a gas-tight stopcock. Each of five reaction tubes held 2.5 ml of high-purity, sulfur dioxide-free water and 110 mg of L-cysteine. The contents of three tubes were pressurized under 15 p.s.i. oxygen while a fourth tube was pressurized with nitrogen (1 p.s.i.=6894.76 Pa). The first three tubes were, respectively, incubated in an oil bath for 20 min at 37, 65 or 100°C and the fourth nitrogen-containing tube was similarly incubated at 100°C. Sulfur dust (1.0 g) was sealed in a fifth tube under 15 p.s.i. N_2–O_2 (80:20) and exposed to 48 h of sunlight at 28°C. The headspace volume (7.0 ml) from each individual tube was collected in a gas-tight syringe and slowly introduced during a 2.0 min period through a 27G needle into 150 μl of 0.05 *M* acetate buffer, pH 5.2 containing OH^{57}CoCbl (e.g., 40 000 cpm). Analytical hydrogen sulfide (Aldrich, Milwaukee, WI, USA) was similarly prepared for analysis. In all cases, the resulting 150-μl volume, containing any OH^{57}CoCbl adducts indicative of headspace volatiles, was administered to a SP-Sephadex C-25 column for RCA as described. Sodium and ammonium sulfides (750 μg/50 μl acetate buffer) were individually admixed with 100-μl volumes of OH^{57}CoCbl to give corresponding sulfide adducts for use as RCA standards.

Bread baking studies that explored normal but

modifiable yeast metabolism of L-cysteine into hydrogen sulfide as a function of assimilable nitrogen, used the following ingredient formulation: active dry yeast (7.0 g); high-purity water (228 g); sucrose (25.0 g); flour (390 g); and L-cysteine (600 mg). Following typical kneading and preliminary rising steps, 65% of the finished dough was rolled, placed in a steel baking pan (12 *w*×22 *l*×8.5 *h* cm), allowed to rise a second time for 35 min, and baked at 180°C for 33 min. Where increased nitrogen was used in the ingredient formulation, ammonium sulfate (3.5 g) was added to the dry ingredient mixture. A core sample of the finished bread was sampled 35 min after baking for RCA studies.

3. Results

3.1. Analytical performance

Earlier reports have already shown the ability of cation-exchange gel chromatography to detect SO_3Cbl in biological materials [9,10,13], but the quantitative reaction of sulfite with OH^{57}CoCbl before radiochromatographic resolution of SO_3^{57}CoCbl has never been reported as a tactic for determining free sulfite levels.

In this study, discriminative RCA of SO_3^{57}CoCbl followed its concentration dependent formation from free sulfite reaction with a uniformly fixed amount of OH^{57}CoCbl (e.g., 40 000 cpm). Using this approach, $\log_{10}$ (abscissa) standard sulfite concentrations, expressed in moles of sulfite anion per 50-μl sample, were plotted against corresponding logit values (ordinate) for each molar sulfite concentration that produced a radiometrically detectable amount of SO_3^{57}CoCbl. The standard regression line used for determining an unknown SO_3^{57}CoCbl concentration was calculated to be $y=1.186x+10.656$, where "*y*" was the logit B/B_0 value and "*x*" represented the $\log_{10}$ of sulfite concentration expressed in moles per 50 μl of sample. The standard deviations (SDs) for the line slope and *y*-intercept were, respectively, ±0.17 and ±0.26 based on triplicate (3) analyses of sulfite standards over seven (7) concentration levels from 0.3 p*M*–6.0 n*M*. The correlation coefficient (*r*) for the three separately run regression lines was 0.96±0.02, and the regression coefficient standard error was 0.079 for the calculated line using all 21 standards. The relative standard deviation (RSD) for radiometric counts of SO_3^{57}CoCbl produced by four independently assayed 6.0 n*M* sulfite standards was 4.4%, while 0.3 p*M* standards gave a 29.5% RSD. An additional study of 6.0 p*M*, 60 p*M*, 600 p*M* and 6.0 n*M* sulfite standards in quadruplicate revealed an overall average 14.6±9.2% RSD. The higher RSD observed at the lowest sulfite concentration was attributable to effects of: (1) separate replicate preparations of individual sulfite standards, plus (2) the construction of new gel columns for each sulfite analysis. These steps were necessary since standard sulfite solutions were unstable after 1.0 h, and the high sensitivity of the method required fresh gel columns to avoid run-to-run cross-contamination effects from one RCA to the next. Run-to-run variations in precision for elution of SO_3^{57}CoCbl at 8000–8160 μl for 10 replicate RCA separations indicated a 1.42±0.25% RSD.

3.2. Application results for sulfite quantification in baked products

Three events involving the consumption of bakery products uniquely underscored the necessity for developing the sulfite radioassay reported here. In the first event, five unrelated subjects complained of foodborne sulfite intolerance effects reminiscent of their previous experiences with sulfited wine, however these effects developed after they had all eaten fresh-bread baked at local markets. A second incident involved a sixth subject who was known to be sulfite intolerant and currently receiving vitamin B_{12} (cyanocobalamin) injections for management of pernicious anemia. This subject collapsed in shock minutes after partial consumption of a warm croissant while still on the site of its purchase. In the third case, a seventh 84-year-old individual with no pharmaceutical intervention for management of Parkinsonian symptoms and no serum cobalamin deficiency, experienced repeated exacerbations of the neurological disorder 10 separate times, at the same time each week. These events followed consumption of a donut obtained at the same venue where the sixth subject purchased the croissant. Moreover, the onset of exacerbated Parkinsonian symptoms ranged from

10–60 min depending on whether or not hot or cold beverages accompanied donut consumption.

Purveyors of bread, croissant and donut products would not confirm sulfite addition to their proprietary dough formulations but some acknowledged their prerogative to use a L-cysteine dough conditioner. Analysis of the questionable baked products with the Monier–William's method [15] and ion-exclusion chromatography–ED [16,17] failed to show evidence of sulfite at a detection limit of 1.0 μg/g. Using the method presented here however, free foodborne sulfite was found in all products as a RCA elution peak indicative of SO_3^{57}CoCbl (Fig. 1). The free sulfite detected in store-baked bread was 0.40±0.01 μg/g; donuts ranged from 0.19±0.01–0.31±0.04 μg/g depending on the bakery; and croissants had levels of 0.24±0.01 μg/g. Products from commercial bakeries that were not linked to intolerance effects included white bread and plain bagels which had respective sulfite levels of 0.029±0.001 and 0.007±0.001 μg/g.

It is reported that baking industry practices commonly rely on activated dough development (ADD) to expedite the functional rheological properties of dough [24,25]. This effect can be simply achieved by adding L-cysteine or sulfite salts to meet product quality specifications. As reducing agents, these additives cleave the disulfide linkages of dough proteins. This action decreases protein average molecular masses and predictably modifies strong flour functionality where light textured pastry and cake-like baked products are desired [24].

Although sulfites and L-cysteine exert similar dough conditioning effects, confirmation of their specific individual uses in baked products remained analytically unresolved. In part, this reflected a paucity of practical analytical data for identifying volatile or nonvolatile byproduct residues of these sulfur-containing additives in final baked products. It has been shown however, that 7% of radioactive hydrogensulfite ($H^{35}SO_3^-$) added to cookie dough gets converted to sulfur dioxide ($^{35}SO_2$) [26]. Some sulfur dioxide residues may persist in the structural voids of low density baked products, and other residues interact with carbohydrates in dough formulations. The latter reaction serves as the basis for blocking the Maillard reaction [27]. In this study, cross-sectional analyses of a commercially prepared donut reinforced the likelihood that sulfur dioxide migration had also occurred during thermal processing. Whereas RCA studies indicated a sulfite level of 0.33±0.03 μg/g for a complete cross-section of a donut product, the top 0.5 cm had only 0.15±0.01 μg/g and the 1.5 cm core had 0.24±0.01 μg/g.

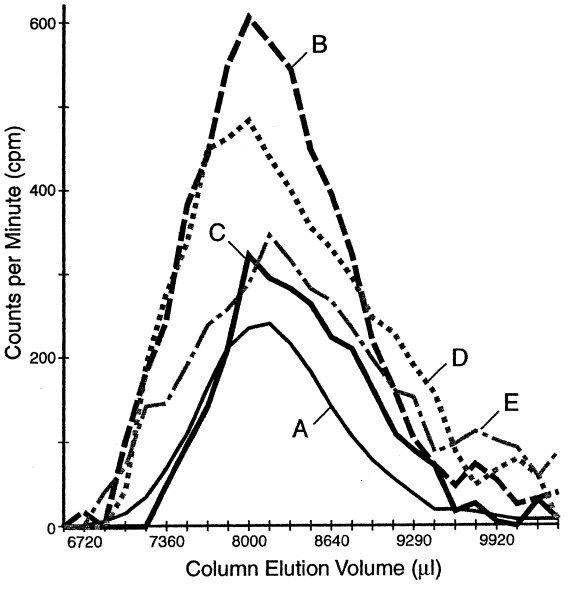

Fig. 1. RCA elution profiles for (A) sulfite standard (480 pg/50 μl sample); and, freshly prepared (B) baked bread, (C and D) plain croissants from different bakeries, and (E) a donut.

3.3. Radiochromatographic characterization of headspace volatiles derived from L-cysteine as sulfur dioxide and sulfides – relationships to other foods

Since RCA resolution of SO_3^{57}CoCbl at 8000–8160 μl was contingent on OH^{57}CoCbl adduct formation with sulfite, it was noted that baked products formulated with L-cysteine also gave a detectable peak in the same elution range (Fig. 2). However, unlike RCAs for sulfite, baked products formulated with L-cysteine showed a 8000–8160 μl elution peak preceded by evidence of one or more OH^{57}CoCbl adducts with elution peaks in the range of 6880–7840 μl. Due to the unknown analytical significance of these elution profiles, many different experimental OH^{57}CoCbl adducts were prepared and

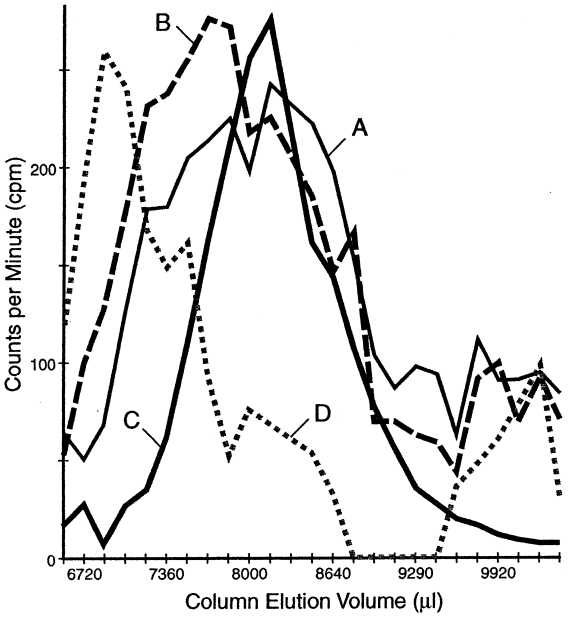

Fig. 2. RCA elution profiles for (A) model bread formulation containing L-cysteine plus ammonium sulfate as an added nitrogen source; (B) model bread formulation with cysteine and no additional nitrogen; (C) diced apple pieces contained in a bagel; and (D) brown raisins removed for analysis from commercially baked and packaged raisin bread.

studied. These efforts were directed by literature accounts that L-cysteine readily undergoes thermal decay with the release of hydrogen sulfide [28,29]. At least some of this sulfide may undergo secondary thermal reactions to yield more complex sulfides, or it may participate in reactions with oxygen or peroxides to produce sulfur dioxide from sulfide [30,31].

Adducts of cobalamins with sulfide(s) have been reported [4] but they have never been viewed with any possible significance to analytical food chemistry. Seven lines of direct and circumstantial evidence supported the contention that OH^{57}CoCbl adducts detected in the elution range of 6560–7840 μl were indicative of sulfides including: (1) headspace volatiles released from L-cysteine at 37, 65 and 100°C under 15 p.s.i. oxygen; (2) headspace volatiles produced from L-cysteine at 100°C under 15 p.s.i. nitrogen; (3) headspace volatiles detected above elemental sulfur in N_2–O_2 (80:20) at 28°C; (4) hydrogen sulfide; (5) sodium sulfide; (6) ammonium sulfide; and (7) L-cysteine formulated yeast doughs supplemented with ammonium nitrogen (Figs. 2–5).

RCA detectable adducts of sulfides generally eluted at ≤8000 μl while sodium sulfide eluted as early as 6880–7040 μl (Fig. 3). Analyses of L-cysteine volatiles produced under *oxygen* at 37 and 65°C gave characteristic elution peaks at 7840 μl yet L-cysteine samples at 37, 65 and 100°C all displayed indications of a SO_3^{57}CoCbl peak at 8000–8160 μl (Fig. 4). The latter peak was consistent with evidence for SO_3^{57}CoCbl prepared from known reactions of OH^{57}CoCbl with sulfite or sulfur dioxide in headspace volatiles existing over elemental sulfur in N_2–O_2 (80:20) (Fig. 3). Identically prepared headspace volatiles from L-cysteine at 100°C under *nitrogen* produced a large radiometric elution peak at 7520 μl (Fig. 4). Observation of this peak was reminiscent of earlier RCA studies where a sulfide species was suggested as a possible OH^{57}CoCbl adduct produced from volatiles above elemental sulfur dust as well as standard hydrogen sulfide (Fig. 3). Furthermore, the relative absence of oxygen with

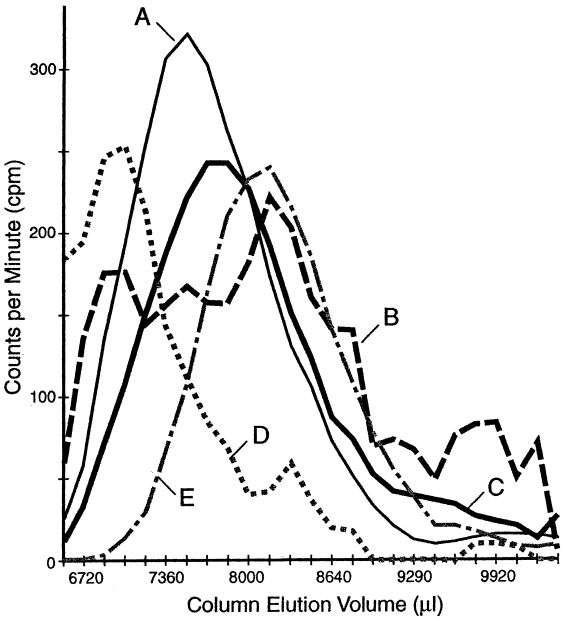

Fig. 3. Elution profiles for various inorganic sulfur standard RCAs including (A) hydrogen sulfide; (B) headspace volatiles originating from elemental sulfur dust in an oxygen-rich atmosphere; (C) ammonium sulfide; (D) sodium sulfide and (E) sodium sulfite.

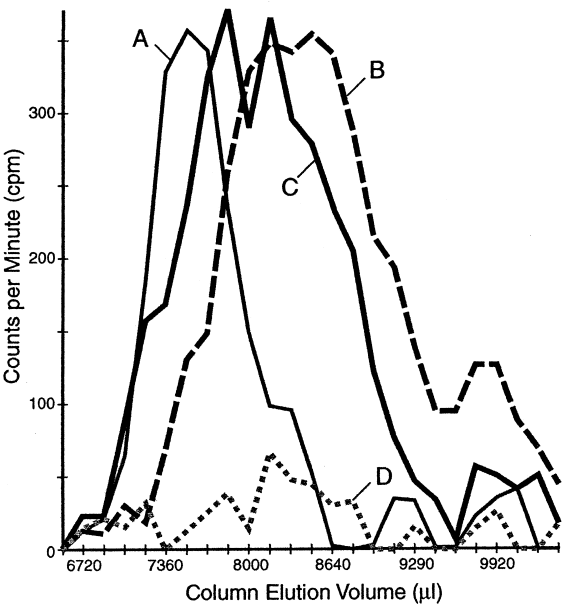

Fig. 4. Chromatographic elution profiles for headspace volatiles detected as $OH^{57}CoCbl$ adducts released from the thermal decomposition L-cysteine at (A, B) 100°C, (C) 65°C and (D) 37°C. Headspace volatiles detected in (B)–(D) were formed from L-cysteine in an oxygen atmosphere while those detected in (A) developed from L-cysteine held under nitrogen at 100°C. Note the absence of any well defined indication of a $SO_3^{57}CoCbl$ elution peak in (A) contrary to headspace volatiles detected in (B)–(D) with the presence of oxygen.

L-cysteine during volatile formation at 100°C, correlated with a dramatic elimination of any sulfur dioxide detected as $SO_3^{57}CoCbl$ at an 8000–8160 μl elution volume (Fig. 4).

The presence of sulfide $OH^{57}CoCbl$ adducts detected in the RCA elution range of 6560–7840 μl, was further supported by idiosyncrasies of yeast metabolism. The biodynamic release of sulfide from L-cysteine is tied to assimilable nitrogen supplied to yeast as an ammonium ion [32]. Low nitrogen levels enhance sulfide formation and the reverse is true. Application of this principle to controlled baking studies using L-cysteine formulated doughs, revealed collateral alterations in RCA profiles for detectable $OH^{57}CoCbl$ adducts. For example, the calculated radiometric (cpm) peak occurrence ratio, for sulfide-sensitive $OH^{57}CoCbl$ adduct elution at 7680–7840 μl compared to $SO_3^{57}CoCbl$ at 8000–8160 μl, was 1.25 for nitrogen deficient yeast leavened bread versus 0.9 for nitrogen supplemented yeast (Fig. 2) and otherwise identical dough formulations.

RCA similarities in $OH^{57}CoCbl$ adducts prepared from L-cysteine headspace volatiles (Fig. 4) were also unexpectedly noticed in studies of imported berries (e.g., strawberries, raspberries and blueberries), strawberry preserves, gingerbread, marshmallows, a specific brand of chicken breast meat and a paperboard ice cream carton (Figs. 5 and 6). All these RCA studies indicated some detectable $SO_3^{57}CoCbl$ at 8000–8160 μl plus one or more coexisting peaks in the range of 6320–7840 μl (Figs. 5–6). Investigations of these samples could not be ignored because they too were uniquely implicated with bouts of exacerbated Parkinsonian symptoms presented by subject 7, quite apart from earlier described insults of donut consumption. Study of the ice cream carton was a special priority since frozen product consumed at the center of the carton had no effect on the individual, yet ice cream in proximity

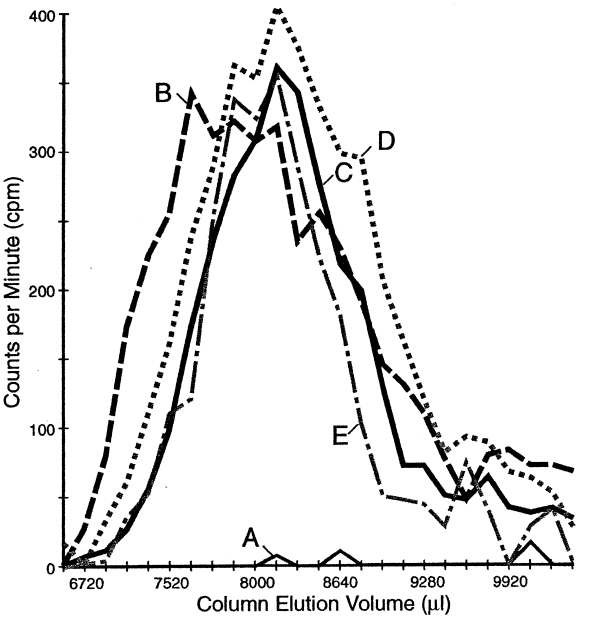

Fig. 5. RCA elution profiles for $OH^{57}CoCbl$ adducts detected in studies of (A) locally grown fresh strawberries and imported (B) strawberries, (C) blueberries, (D) raspberries and (E) commercially prepared strawberry preserves. Identical acetate buffer extracts of 25.0 g food samples (3:1, w/w) were prepared as detailed in the text, and a 0.22 μm filtered, 50-μl volume was subjected to RCA.

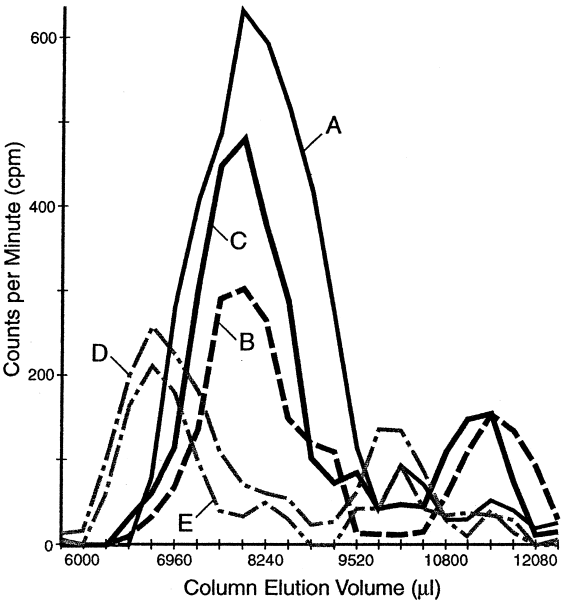

Fig. 6. RCA elution profiles for detectable adducts of $OH^{57}CoCbl$ indicating sulfite presence in gingerbread (A), marshmallows (B), and a croissant (C) that contained an almond paste filling. Analytical elution profiles for extracts of chicken broth (D) and a cardboard ice cream container (E) show similar evidence of sulfides in the 6000-μl elution range. Note the trace detectability of cyanide as $CN^{57}CoCbl$ at elutions of ~11 000 μl where it was contributed by almond paste (C) or integumentary residues present in gelatin, which was used as a marshmallow ingredient (B).

to the paperboard container (Fig. 6) seriously exacerbated Parkinsonian symptoms.

4. Discussion

The merger of quantitative radiometric saturation analysis with a RCA strategy allowed foodborne sulfite detection and quantification as detailed above. The entire protocol was predicated on the ability of $OH^{57}CoCbl$ to form an adduct with free sulfite to give $SO_3^{57}CoCbl$ that was radiochromatographically resolved at an elution volume of 8000–8160 μl. Since this research principally dealt with bakery products implicated in sulfite intolerances of several individuals, any origins of detectable sulfite were uncertain since L-cysteine or sulfite may have been used singularly or in combination with each other to achieve final product objectives. This issue was analytically addressed by noting key RCA differences in detectable $OH^{57}CoCbl$ adducts produced by thermal decay products of L-cysteine as opposed to sulfite. Whereas sulfites produced an RCA elution peak at 8000–8160 μl, L-cysteine gave a similar elution peak accompanied by one or more incompletely resolved minor elution peaks in the range of 6880–7840 μl. Elution peaks in this range were notable for their similarity to standard elution profiles for sodium sulfide, hydrogen sulfide, volatile elemental sulfur compounds (Fig. 3), and biodynamic indications of sulfide eluting at 7840 μl, which developed from yeast in response to L-cysteine with inadequate assimilable nitrogen (Fig. 2). Furthermore, L-cysteine volatiles released at 100°C under nitrogen gave a diminutive peak for $SO_3^{57}CoCbl$ at 8000–8160 μl coincidental with the appearance of a large elution peak reminiscent of sulfide standards at 7520 μl. The identical L-cysteine reaction conducted under oxygen at 100°C gave an impressive indication of RCA detectable $SO_3^{57}CoCbl$ but little evidence of $OH^{57}CoCbl$ adducts which corresponded to known sulfide standards. This would seem to suggest that a variety of sulfur oxides may be detected as RCA adducts.

Thermal decomposition of L-cysteine with the release of hydrogen sulfide has been modeled [28,29], but there is little data regarding the contribution of hydrogen sulfide to sulfur dioxide development in foods during usual heat processing steps. The reaction of cobalamin with sulfide has also been substantiated [4], but the structural varieties of sulfides capable of producing detectable $OH^{57}CoCbl$ adducts are unknown.

Once putative suspicions for sulfite occurrences in bakery products were validated, there was an ancillary focus on foods that exacerbated Parkinsonian symptoms of subject 7. The RCA elution characterizations for L-cysteine volatiles closely resembled those observed in most of these samples. That is, an obvious $SO_3^{57}CoCbl$ elution at 8000–8160 μl was also preceded by one or more elution peaks regarded as sulfide adducts of $OH^{57}CoCbl$. This was true for all berry samples or products studied (Fig. 5). Contrary to imported berries, the consumption of locally grown strawberries failed to produce any serious consequences, and only trace indications of detectable $OH^{57}CoCbl$ adducts were apparent. In-

quiries directed to the producers of imported berries failed to discount the use of antifungal sulfur dusts during fruit cultivation, and another producer blamed any sulfite residues in blueberries on sulfur dioxide impregnated shipping pallets. Whatever the case, the RCA elution profile for detectable OH^{57}CoCbl adducts produced by headspace volatiles above sulfur dust (Fig. 3) and L-cysteine (Fig. 4) were not unlike those analytical results for various berries including strawberry preserves (Fig. 5). Low levels of sulfides have been reported in cultivated strawberries but their origin has never been established [33]. It may be significant that fungicidal sulfur exerts its fumigant action as a penetrating vapor phase above 22°C which can permeate fruit tissue [34]. Beyond this, its fungicidal action also involves the conversion of sulfur to hydrogen sulfide as well as nondescript polysulfide free radicals [35]. In view of these accounts, plus the precedented RCA elution profiles for L-cysteine volatiles, it is possible that evidence of SO_3^{57}CoCbl and suspected sulfide adducts of OH^{57}CoCbl resulted from agricultural sulfur residues.

Sulfured molasses proved to be the source of sulfite detected in gingerbread (0.32 ± 0.01 μg/g) and sodium hydrogensulfite was traced to detectable sulfite in marshmallows (0.04 ± 0.01 μg/g) where it probably ensured a white appearance (Fig. 6). Analysis of the paperboard ice cream carton revealed the notable occurrence of an OH^{57}CoCbl adduct consistent with the previous detection of sodium sulfide at 6880–7040 μl (Figs. 6 and 3). Sulfides, sulfur dioxide and calcium hydrogensulfite have various roles in the digestion of lignin, wood pulp digestion and paper bleaching that may explain origins of the RCA elution profiles detected here. The actual migration of residual sulfur compounds into ice cream from its paper carton may account for the food intolerance experienced by subject 7 since only ice cream close to the paper container produced intolerance effects. In addition, inspection of Fig. 6 also shows the RCA profile for OH^{57}CoCbl adducts present in a water broth that resulted from boiling one unique brand of chicken. A notable OH^{57}CoCbl adduct attributed to sulfides was obvious at a 6880 μl elution volume along with a relatively small detectable SO_3^{57}CoCbl peak at 8000–8160 μl. It was coincidentally interesting that OH^{57}CoCbl adducts detected in acetate buffer extracts of brown raisins (Fig. 2), also indicated sulfide residues in an elution profile nearly identical to that of the paper carton and chicken broth (Figs. 2, 3 and 6).

5. Conclusion

Many methods exist for quantifying foodborne sulfite that satisfy regulatory compliance statutes governing their food additive uses [18]. Sensitivities of these methods may be inadequate however, for exploring the biochemical mechanisms underlying sulfite hyperintolerance, defining no effect levels (NOELs) for sulfite additive use [27], or determining the naturally occurring sulfite residues in foods.

With a pK_1 of 1.7 between sulfurous acid (H_2SO_3) and the hydrogensulfite anion (HSO_3^-) as well as a pK_2 of 7.0 between hydrogensulfite and the sulfite ion (SO_3^{2-}), the hydrogensulfite anion clearly prevails over a pH range of 3.5–7.0 for most foods. Thus, the radioanalytical method described only detects sulfite concentrations freely soluble in pH 5.2 buffer when OH^{57}CoCbl reacts with HSO_3^- to produce SO_3^{57}CoCbl according to the steps defined by Farquharson and Adams [11]. These freely soluble and quantifiable sulfite levels cannot be interpreted as an equivalent measure of sulfite characterized by usual methods that employ pH extremes to liberate all sulfite [15–17].

Quantitative sensitivity of the sulfite radioassay in this study relied on the specific activity of the ^{57}Co-labeled cobalamin, and the selected dilution level of OH^{57}CoCbl (e.g., 40 000 cpm) framed the analytical range for quantifying unknown sulfite levels in the context of a standard logit plot. Many samples were suited for sulfite radioassay provided that OH^{57}CoCbl levels, required for adduct formation, were not adversely affected by sample-specific: R-proteins; particulate adsorption; lipid residues; interaction with high-molecular-mass molecules such as dextrins; microemulsion or hydrocolloidal entrainment; strong ionic strengths (>0.15 M) or pH extremes. Also, since the formation constant for sulfite reaction with OH^{57}CoCbl is second only to that for cyanide [3], SO_3^{57}CoCbl formation supersedes that of other possible OH^{57}CoCbl adduct forms, including known data for sulfide adduct formation.

Thus, the coexistence of sulfides with sulfite did not preclude primary detection of free sulfite. Interference from cyanide may be an analytical concern for some environmental, plant and animal tissue extracts but its quantitative effect can be overcome by uniformly increasing the amount $OH^{57}CoCbl$ for constructing standard logit plots as well as sample analyses. The RCA detection of cyanide as $CN^{57}CoCbl$ typically occurs at an elution volume of ~11 000 μl as seen in Fig. 6 where cyanide residues in a croissant developed from cyanogenic glycosides [27] in an almond paste ingredient. This was consistent with the previous analytical performance of the RCA method where peak elution volumes for $SO_3^{57}CoCbl$, $NO_2^{57}CoCbl$ and $CN^{57}CoCbl$ were, respectively, 8000, 9600 and 10 900 μl [10].

The selective picogram quantification of free sulfites in 50-μl sample volumes represented only one analytical advantage of the method reported. A second merit centered on its ability to quantify foodborne sulfites that were immediately soluble in only mild pH 5.0, 0.05 *M* acetate buffer. Unlike other reported sulfite analytical rationales, measurement of biologically reactive sulfite relied on radiometric $SO_3^{57}CoCbl$ detection by exploiting the natural ligand binding property of $OH^{57}CoCbl$ with free sulfite anions. This approach could be important since it has been suggested that measures of free sulfite may permit a better understanding of foodborne sulfite hyperintolerance than total sulfite [36]. A third unique feature of the method involved its sensitive and discriminative detection of sulfides that coexisted with sulfites. Notwithstanding this discrimination, characteristic food similarities associated with dietary insults to the neurological status of one individual would not have been noticed. Moreover, foods that elicited these responses exhibited RCA profiles for $OH^{57}CoCbl$ adducts first noticed as volatiles released from L-cysteine at only 37°C. The possible involvement of L-cysteine in neurodegenerative scenarios has been widely reported [37–39] but its neuro-disruptive mechanisms are poorly understood. Unfortunately, the low level chronic dietary toxicity effects of sulfite [10] and sulfides on (1) existing or marginal neurological disorders [40–47]; (2) aged membrane performance [48]; and, (3) neurological nitric oxide signaling dynamics remain to be established [49,50]. Continued development, application and interpretation of this sulfite radioassay may afford a better integrated analytical understanding of food intolerances in cases where sulfides seem to commonly coexist with dietary sulfites.

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References

[1] J.A. Hill, J.M. Pratt, R.J.P. Williams, J. Theor. Biol. 3 (1962) 423.
[2] D.H. Dolphin, A.W. Johnson, N. Shaw, Nature 199 (1963) 170.
[3] R.A. Firth, H.A.O. Hill, J.M. Pratt, R.G. Thorp, R.J.P. Williams, J. Chem Soc. A (1969) 381.
[4] E.A. Kaczka, D.E. Wolf, F.A. Kuehl, K. Folkers, J. Am. Chem. Soc. 73 (1951) 3569.
[5] J.M. Buchanan, H.L. Elford, R.E. Loughlin, B.M. McDougall, S. Rosenthal, Ann. NY Acad. Sci. 112 (1964) 756.
[6] R. Ertel, N. Brot, R. Taylor, H. Weissbach, Arch. Biochem. Biophys. 126 (1968) 353.
[7] W.A. Fenton, L.M. Ambani, L.F. Rosenberg, J. Biol. Chem. 251 (1976) 6616.
[8] E.M. Scheuring, I. Sagi, M.R. Chance, Biochemistry 33 (1994) 6310.
[9] J.A. Begley, C.A. Hall, in: B. Zagalak, W. Friedrich (Eds.), Vitamin B_{12}: Proceedings of the 3rd European Symposium on Vitamin B_{12} and Intrinsic Factor, Zurich, March 1979, Walter de Gruyter, Berlin, 1979, p. 821.

[10] J.M. Anes, R.A. Beck, J.J. Brink, R.J. Goldberg, J. Chromatogr. B 660 (1994) 180.
[11] J. Farquharson, J.F. Adams, Am. J. Clin. Nutr. 30 (1977) 1617.
[12] Y. Kobayashi, D.V. Maudsley, Biological Applications of Liquid Scintillation Counting, Academic Press, New York, 1974.
[13] J.A. Begley, C.A. Hall, J. Chromatogr. 177 (1979) 360.
[14] G. Tortolani, P. Bianchini, V. Mantovani, J. Chromatogr. 53 (1970) 577.
[15] Official Methods of Analysis, AOAC, Arlington, VA, 14th Edition, Sections 20.123–20.125, 1984.
[16] H.-J. Kim, G.Y. Park, Food Technol. 41 (1987) 85.
[17] H.-J. Kim, K.R. Conca, M.J. Richardson, J. Assoc. Off. Anal. Chem. 73 (1990) 983.
[18] J.W. DeVries, C.R. Warner, Food Testing Anal. 3 (1997) 22.
[19] H.-J. Kim, J. Assoc. Off. Anal. Chem. 72 (1989) 266.
[20] H.-J. Kim, Y.-K. Kim, J. Food Sci. 51 (1986) 1360.
[21] J.F. Kolhouse, H. Kondo, N.C. Allen, E. Podell, R.H. Allen, New Engl. J. Med. 299 (1978) 785.
[22] E. Mortenson, Clin. Chem. 18 (1972) 895.
[23] R.A. Beck, J. Food Sci. 44 (1979) 1077.
[24] C.S. Fitchett, P.J. Frazier, in: J.M.V. Blanshard, P.J. Frazier, T. Galliard (Eds.), Proceedings of an International Symposium, Food Chemistry Group of the Royal Society of Chemistry and the School of Agriculture of the University of Nottingham, South Bonington, April 1985, Royal Society of Chemistry, London, 1985, p. 179.
[25] C.E. Stauffer, Functional Additives for Bakery Foods, Avi/Van Nostrand Reinhold, New York, 1990.
[26] B.H. Thewlis, P. Wade, J. Sci. Food Agric. 25 (1974) 99.
[27] C. Zapsalis, R.A. Beck, in: Food Chemistry and Nutritional Biochemistry, Wiley, New York, 1985, p. 123.
[28] Y. Zhang, M. Chien, C.-T. Ho, J. Agric. Food Chem. 36 (1988) 992.
[29] C. Macku, T. Shibamoto, J. Agric. Food Chem. 39 (1991) 1987.
[30] E.D. Weil, in: Kirk–Othmer Encyclopedia of Chemical Technology, Vol. 22, Wiley, New York, 1983, p. 107.
[31] L. Pauling, in: General Chemistry, W.H. Freeman, San Francisco, CA, 1970, p. 268.
[32] V. Jiranek, P. Langridge, P.A. Henschke, Appl. Environ. Microbiol. 61 (1995) 461.
[33] P.J. Dirinck, H.L. De Pooter, G.A. Willaert, N.M. Schamp, J. Agric. Food Chem. 29 (1981) 316.
[34] G.W. Ware, Pesticides: Theory and Application, W.H. Freeman, New York, 1983.
[35] C.M. Menzie, in: Metabolism of Pesticides, US Department of Interior, Special Scientific Report, Wildlife No. 127, US Department of Interior, Washington, DC, 1969.
[36] Anonymous, Food Chem. News, 19 Nov. (1984) 19.
[37] M.T. Heafield, S. Fearn, G.B. Steventon, R.H. Waring, A.C. Williams, S.G. Sturman, Neurosci. Lett. 110 (1990) 216.
[38] J.W. Olney, C. Zorumski, M.T. Price, J. Labruyere, Science 248 (1990) 596.
[39] A. Slivka, G. Cohen, Brain Res. 608 (1993) 33.
[40] K. Abe, H. Kimura, J. Neurosci. 16 (1996) 1066.
[41] N. Inoue, Nippon Rinsho 11 (1993) 2924.
[42] M.F. Beal, Ann. Neurol. 44 (1998) 3110.
[43] J.K. Shergill, R. Cammack, C.E. Cooper, J.M. Cooper, V.M. Mann, A.H. Schapira, Biochem. Biophys. Res. Commun. 228 (1996) 298.
[44] A.A. Olkowski, Vet. Hum. Toxicol. 39 (1997) 355.
[45] R. Hosoki, N. Matsuki, H. Kimura, Biochem. Biophys. Res. Commun. 237 (1997) 527.
[46] J.C. Lavoie, P. Chessex, Pediatr. Res. 33 (1993) 347.
[47] S.B. Harvey, G.L. Nelsestuen, Biochim. Biophys. Acta 1267 (1995) 41.
[48] M.C.C. Lizada, S.F. Yang, Lipids 16 (1981) 189.
[49] J.S. Stamler, J.A. Osborne, O. Jaraki, L.E. Rabbani, M. Mullins, D. Singel, J. Loscalzo, J. Clin. Invest. 91 (1993) 308.
[50] S.A. Lipton, P.A. Rosenberg, New Engl. J. Med. 330 (1994) 613.