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Review

Safety evaluation of substituted thiophenes used as flavoring ingredients

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ABSTRACT

This publication is the second in a series by the Expert Panel of the Flavor and Extract Manufacturers Association summarizing the conclusions of its third systematic re-evaluation of the safety of flavorings previously considered to be generally recognized as safe (GRAS) under conditions of intended use. Re-evaluation of GRAS status for flavorings is based on updated considerations of exposure, structural analogy, metabolism, pharmacokinetics and toxicology and includes a comprehensive review of the scientific information on the flavorings and structurally related substances. Of the 12 substituted thiophenes reviewed here, 11 were reaffirmed as GRAS based on their rapid absorption, metabolism and excretion in humans and animals; the low estimated dietary exposure from flavor use; the wide margins of safety between the conservative estimates of intake and the no-observed-adverse effect levels; and the lack of significant genotoxic and mutagenic potential. For one of the substituted thiophenes, 3-acetyl-2,5-dimethylthiophene, it was concluded that more detailed exposure information, comparative metabolism studies and comprehensive toxicity data, including an in-depth evaluation of the mechanism of action for any adverse effects observed, are required for continuation of its FEMA GRAS™ status. In the absence of these data, the compound was removed from the FEMA GRAS list.

Contents

Abbreviations: bw, body weight; CYP, cytochrome P450; DNA, deoxyribonucleic acid; DT, decision tree; F, female; FEMA, the Flavor and Extract Manufacturers Association; GLP, good laboratory practice; GRAS, generally recognized as safe; GRASa, GRAS affirmed; GRASr, GRAS reaffirmed; GSH, glutathione; *i.p.*, intraperitoneal; LD_{50}, median lethal dose; JECFA, Joint FAO/WHO Expert Committee on Food Additives; M, male; MNBN cells, micronucleated binucleate cells; MS, mass spectrometry; MSDI, maximized survey-derived intake; MTD, maximum tolerated dose; NA, data not available; NAS, National Academy of Science; NMR, nuclear magnetic resonance; NOAEL, no-observed-adverse effect level; NOEL, no-observed-effect level; NTP, National Toxicology Program; PADI, possible average daily intake; ppm, parts per million; S-9, metabolic activation system; TTC, threshold of toxicological concern.

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1. Introduction

Following the adoption of the 1958 Food Additive Amendment to the Food, Drug and Cosmetic Act (FDCA), the Flavor and Extract Manufacturers Association (FEMA) established the "generally recognized as safe" (GRAS) Program to evaluate the safety of food flavor ingredients based on scientific data. The safety of flavor ingredients is determined by the FEMA Expert Panel, a body of independent scientists in fields of biochemistry, toxicology and medicine who serve as reviewers of scientific data related to the safety of flavor ingredients (Smith et al., 2005a). The GRAS status of flavor ingredients is re-affirmed periodically as part of ongoing FEMA GRAS re-evaluations, a key component of the FEMA GRAS Program. Re-evaluations are prioritized when there is a significant increase in exposure, or a substantial body of new scientific data that has become available since the previous evaluation.

This paper is the second publication (following Marnett et al., 2014) from the third cycle of re-evaluations of the GRAS status of flavoring substances. It represents the Expert Panel's re-evaluation of the GRAS status of flavoring substances that belong to the group of substituted thiophenes, five-member aromatic heterocycles with sulfur as the only heteroatom in the ring. The current re-evaluation was initiated by the availability of new toxicity data, particularly genotoxicity data for a number of substances in the group, and new data on the metabolism of other representative substituted thiophenes.

2. Chemical identity

This group of twelve flavoring agents (Table 1) includes thiophene derivatives, which are five-membered heterocycles containing only sulfur as the ring heteroatom. The substances in this group are all ring-substituted with one or more of the following substituents or functional groups: aliphatic (3), thioether (1), disulfide (1), alkyl thiol (2), alkyl alcohol (1) and alkyl ketone (4) moieties (Table 1).

The structural class of the 12 thiophenes presented here was determined based on the Decision Tree (DT) criteria (Cramer et al., 1978), with 7 substances assigned to structural class II (FEMA Nos 3209, 4137, 4142, 4387, 4642, 4643, 4645) and 4 substances to class III (FEMA Nos 3062, 3323, 4184, 4646). The compound 3-acetyl-2,5-dimethylthiophene (previously FEMA No 3527) was assigned to structural class II.

3. Status as flavoring substances

Of the group of 12 substituted thiophene substances, four members, 2-thienyl mercaptan, 5-methyl-2-thiophenecarboxaldehyde, 2-thienyldisulfide and 3-acetyl-2,5-dimethylthiophene were originally assigned their FEMA GRAS status as part of the GRAS 3, 4, 5 and 11 publications, respectively (Hall and Oser, 1965, 1970; Oser and Hall, 1972; Oser and Ford, 1978). Subsequently, these substances were re-evaluated by the Expert Panel in 2001 and reaffirmed as GRAS under conditions of use as flavoring ingredients. The remaining eight members of this

group were determined to be GRAS under conditions of intended use more recently (2005–2009) (Smith et al., 2005b, 2009; Waddell et al., 2007).

All 12 substances in the group have also been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as part of the group of sulfur heterocycles and structurally related flavoring ingredients, and the Committee concluded the substances would not present a safety concern at the current levels of intake (JECFA, 2002, 2007, 2012). In addition, two of the substances in this group, 2-pentylthiophene and 2-hexylthiophene, were also evaluated by EFSA in 2013 among a group of sulfur heterocycles for use as flavor ingredients under EFSA's regulatory jurisdiction. EFSA concluded that additional toxicity data were required (these have become available and are presented here) (EFSA, 2013a,b). In the same EFSA evaluation, another four substances of this group, 2-thienyl mercaptan, 5-methyl-2-thiophenecarboxaldehyde, 2-thienyl disulfide and 3-acetyl-2,5-dimethylthiophene were included as supporting substances (EFSA, 2013a). Two substances, 5-methyl-2-thiophenecarboxaldehyde and 3-acetyl-2,5-dimethylthiophene, were evaluated for genotoxicity by EFSA in 2013 (EFSA, 2013c–e). Of these two, 5-methyl-2-thiophenecarboxaldehyde was concluded to be safe for use as a flavoring substance in food (EFSA, 2013c). Finally, EFSA concluded that one substance that was previously evaluated by JECFA was safe for use as a flavoring substance in a separate opinion in 2013 (EFSA, 2013f).

4. Exposure

Thiophene derivatives are common components of food or are structurally related to common components of food. They occur naturally in a variety of foods including coffee, beef, pork, shrimp, papaya and whiskey (Nijssen et al., 2015). Thiophenes are also formed during cooking either *via* non-enzymatic browning reactions involving S-containing amino acids (*e.g.* cysteine) or alternatively, by the initial formation of hydrogen sulfide from the sulfur-containing amino acids followed by reactions of hydrogen sulfide with browning intermediates (Heath and Reineccius, 1986).

Annual volumes of production for these ingredients and the daily per capita intake values calculated using the maximized survey-derived intake (MSDI) method are summarized in Table 1. From the most recent annual poundage surveys of 2005 and 2010, the annual reported volume of thiophenes used as added flavoring ingredients to food in the US has been minimal (the highest reported volume was 1 kg/year) (Gavin et al., 2008; Harman et al., 2013). Consequently, the estimated daily per capita intake using the MSDI method has remained very low (see Table 1). The annual production volume has been reduced for at least two substances in the group, 2-thienyldisulfide (from 0.5 to 0 kg/year) and 2-hexylthiophene (from 1 to 0.1 kg/year). Four substances from this group [2-thienylmethanol, 2-acetyl-5-methylthiophene, 3,4-dimethylthiophene, and 1-(2-thienyl)ethanethiol] were recently introduced as flavoring ingredients (2009) and have only been included in the most recent poundage survey (Harman et al., 2013). The most recent poundage survey also includes one other GRAS substance, 2-pentylthiophene that was not previously reported (Table 1).

The flavor effect that the thiophenes impart explains the low volume and use levels as added flavor ingredients in food. Thiophenes often have a strong organoleptic profile, such as burnt caramel, roasted coffee, nutty, earthy, meaty, grassy, roasted onion, as well as softer floral fruity effects, and are used as flavor ingredients at average usual use levels of 0.001–2 ppm in a range of food categories (Hall and Oser, 1965, 1970; Oser and Hall, 1972; Oser and Ford, 1978; Smith et al., 2005b, 2009; Waddell et al., 2007). They are most commonly used in baked goods, beverages, breakfast cereals, candies, cheese, condiments, egg products, fats and oil, fish products, frozen dairy products, gravies, instant coffee, meat products, poultry, processed and reconstituted vegetables, soups, snack foods, and gelatins and puddings (exceptional uses have included up to 250 ppm in seasonings) (Smith et al., 2005b).

While seven of the thiophene derivatives in this flavor group have been detected in a broad range of foods, quantitative natural occurrence data are available only for four substances, namely 5-methyl-2-thiophenecarboxaldehyde, 2-thienylmethanol, 2-pentylthiophene, and 2-acetyl-5-methylthiophene (Nijssen et al., 2015). The estimated annual consumption of these flavor ingredients from natural sources are 1426 kg, 7 kg, 2736 kg and 1165 kg, respectively (Table 1) (Stofberg and Grundschober, 1987). The lack of additional quantitative data results in underestimation of the exposure from sources where they occur naturally. Therefore, consumption ratios can only partially be estimated and indicate that exposure to the naturally occurring substances from ingestion of traditional foods (even though data are only partially available) is expected to be significantly higher than the exposure from their use as added food flavors (i.e., consumption ratio substantially higher than 1) (Table 1).

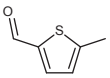
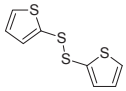
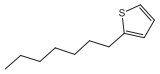
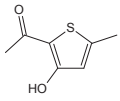
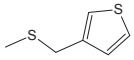
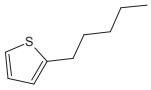
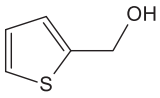
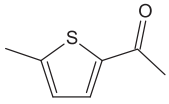
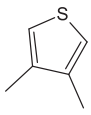
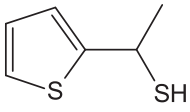
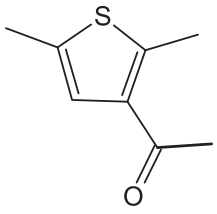
5. Absorption, distribution, metabolism and elimination

The metabolic fate of thiophenebased substances has been studied in detail for a small number of substituted thiophene derivatives that have been used as model structures to clarify differences in metabolic pathways based on specific structural features. In addition, the metabolic pathways involved in the transformation of thiophene derivatives were recently reviewed for a number of xenobiotic compounds containing a thiophene substructure (Gramec et al., 2014). The proposed metabolic pathways in that comprehensive review are consistent with those presented here. From these publications, it is noteworthy that the metabolic transformation of thiophenes is a key element of their potential biological activity and potential toxicity through the formation of reactive intermediates (Gramec et al., 2014).

Common features of metabolism that have emerged in these studies indicate that the majority of the 12 flavoring ingredients are expected to be subject to similar metabolic transformations. Based on the body of evidence from published *in vitro* and *in vivo* metabolic studies, substituted thiophenes are subject to biotransformation *via* oxidative reactions including, S-oxidation or ring epoxidation/hydroxylation. These reactions are followed by conjugation with glutathione (GSH) on the ring and subsequent urinary elimination of mercapturic acids (these are described in detail below and shown in Fig. 3). Structural features of the thiophene ring, including a) the presence and number of substitution groups, b) the type of substitution groups, e.g., alkyl or acyl side chains, and c) the location of substitution groups on the ring, determine the relative contribution of the possible metabolic pathways.

Specifically, alkyl-substituted thiophenes (related thiophene flavoring ingredients include FEMA numbers 4137, 4387, 4642, and 4646) including those with sulfur-containing side chains (related FEMA numbers include 3062, 3323, 4184, and 4645) are expected to undergo primarily ring epoxidation and to a lower extent, S-oxidation; alkyl side chains and sulfur-containing side chains have less contribution to electron rearrangement following ring oxidation relative to acyl side chains (related FEMA numbers 3209; 4142; 4643; and 3-acetyl-2,5-dimethylthiophene); 2-acyl substituted thiophenes are expected to preferentially undergo ring epoxidation (related FEMA numbers 3209; 4142; 4643); 3-acyl substituted thiophenes are expected to preferentially undergo S-oxidation (3-acetyl-2,5-dimethylthiophene) (Table 2). Relative to the other thiophenes, 3-acetyl-2,5-dimethylthiophene contains unique

Table 1
Identity and exposure data for thiophenes and structurally related substances used as flavoring ingredients.

Flavoring ingredient	FEMA No.	DT Class[a]	CAS No. and Structure	Most recent annual volume[b]; kg	Daily per capita Intake[c] ("eaters only")		Annual volume in naturally occurring foods[d]; kg	Consumption Ratio[e]
					μg/d	μg/kg bw/d		
2-Thienyl mercaptan	3062	III	7774-74-5 	0.1	0.01	0.0002	–	NA
5-Methyl-2-thiophenecarboxaldehyde	3209	II	13679-70-4 	1	0.1	0.002	1426	1426
2-Thienyldisulfide	3323	III	6911-51-9 	0.5 (2008)	0.06	0.001	–	NA
2-Hexylthiophene	4137	II	18794-77-9 	0.1	0.01	0.0002	+	NA
1-(3-Hydroxy-5-methyl-2-thienyl)ethanone	4142	II	133860-42-1 	0	NA	NA	–	NA
3-(Methylthio)-methylthiophene	4184	III	61675-72-7 	0	NA	NA	–	NA
2-Pentylthiophene	4387	II	4861-58-9 	0.1	0.01	0.0002	7	70
2-Thienylmethanol	4642	II	636-72-6 	0	NA	NA	2736	NA
2-Acetyl-5-methylthiophene	4643	II	13679-74-8 	0	NA	NA	1165	NA
3,4-Dimethylthiophene	4645	II	632-15-5 	0	NA	NA	+	NA
1-(2-Thienyl)ethanethiol	4646	III	94089-02-8 	0	NA	NA	–	NA
3-Acetyl-2,5-dimethylthiophene	No longer GRAS	II	2530-10-1 	0	0	0	+	NA

[a] Cramer et al., 1978.
[b] From Harman et al., 2013 for materials 3062–4387 and from interim industry survey for materials 4642–4646. Values greater than zero but less than 0.1 kg were reported as 0.1 kg. The values are zero if reported as zero in current and/or previous surveys.
[c] MSDI (μg/person/day) calculated as follows: [[(annual volume, kg) x (1 × 109 μg/kg)]/[population x survey correction factor x 365 days]], where population (10%, "eaters only") = 31 × 106 for the USA, where the correction factor = 0.8, representing the assumption that only 80% of the annual flavor volume was reported in the poundage surveys (Harman et al., 2013). [(μg/person/day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.
[d] Quantitative data for the United States calculated using currently available natural occurrence concentrations, according to methods reported by Stofberg and Grundschober (1987); "+" indicates reported qualitative evidence of natural occurrence without quantitative data.
[e] The consumption ratio is calculated as follows: (annual consumption *via* food, kg)/(most recent reported volume as a flavoring substance, kg).

features that set it apart from the rest of the group of thiophene flavoring ingredients. From the patterns that have emerged with model thiophene structures, all the members of the thiophene flavoring ingredients group with the exception of 3-acetyl-2,5-dimethylthiophene are expected to be metabolized to non-reactive intermediates and/or be efficiently conjugated and readily excreted.

Several cytochrome P450 (CYP) enzymes and related monooxygenases (sensitive to metapyrone and diethylaminoethyl 2,2-diphenylvalerate with EC_{50} of 10 μM, but distinct from flavin monooxygenases and epoxide hydrolase) are involved in ring oxidation reactions of model thiophenes (Dansette et al., 1990). In particular, CYP1A1 (Dansette et al., 2005) and CYP2C9 (Rademacher et al., 2012) are directly involved in oxidation of substituted thiophenes and metabolic activation *in vitro*. Following oxidation reactions and electron rearrangement, most metabolic intermediates of substituted thiophenes are converted to stable hydrophilic products that are anticipated to be efficiently conjugated and excreted. A key factor in the efficient deactivation of metabolic intermediates and the formation of stable final metabolic products and their elimination is the abundant availability of nucleophiles such as GSH. Possible molecular mechanisms of thiophene oxidation and conjugation with GSH have been proposed (Valadon et al., 1996; Medower et al., 2008; Dansette et al., 2009; Guengerich and Isin, 2014).

The thiophene structures shown in Fig. 1 provide insights into the possible metabolic pathways of substituted thiophenes and are used as models for the metabolic fate of the thiophene flavoring substances in this evaluation.

5.1. Unsubstituted thiophene ring metabolism

Unsubstituted thiophene is biotransformed *in vivo* to mercapturic acid metabolites *via* S-oxidation reactions (Dansette et al., 1992). In male Sprague-Dawley rats injected (i.p.) with 200 mg/kg ^{3}H-thiophene, approximately 31% of the radioactivity was excreted in the urine within 15 h and an additional 4% within 50 h (Dansette et al., 1992). The primary urinary metabolite, accounting for more than 94% of the urinary radioactivity and 30% of the administered dose, was the 2-mercapturic acid derivative of 2,5-dihydrothiophene-S-oxide (dihydrothiophene sulfoxide mercapturate; DHTSM) (Fig. 2). These results indicate the formation of a reactive S-oxide intermediate followed by a Michael-type addition of GSH on position 2 of the thiophene ring, rather than an arene epoxide intermediate, which would be consistent with the formation of 3-hydroxydihydrothiophene mercapturate (HDHTM) (Fig. 2). The formation of other biotransformation products cannot be excluded since this study did not account for the fate of the total dose of parent compound administered. Previous evidence indicates that approximately 32% of administered thiophene may be exhaled unchanged (Bray et al., 1971).

5.2. Substituted thiophenes metabolism

5.2.1. S-Oxidation

Substituted thiophenes undergo S-oxidation and a Michael-type addition of sulfur nucleophiles on ring position 2 of the activated thiophene sulfoxide intermediate based on studies *in vitro* and *in vivo* (Mansuy et al., 1991; Valadon et al., 1996). Benzothiophene and [2,3-dichloro-4-(thiophene-3-carbonyl)phenoxy]acetic acid (tienilic acid isomer, TAI) were incubated with activated liver microsomal fractions *in vitro* in the presence of NADPH and mercaptoethanol (100 μM) (Mansuy et al., 1991). The final products, the 3-thioethanol conjugate of benzothiophene sulfoxide and the 2-thioethanol conjugate of TAI sulfoxide, respectively, indicate the formation of a S-oxide reactive intermediate for both structures (Mansuy et al., 1991). The presence of an acyl substituent plays a key role in the reactivity towards thiol nucleophiles, rendering the S-oxide of TAI (bearing a keto substituent) more reactive than the S-oxide of benzothiophene (lacking an acyl substituent) (Mansuy et al., 1991).

5.2.2. Ring epoxidation

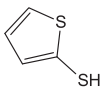
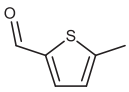
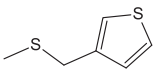
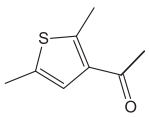
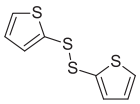
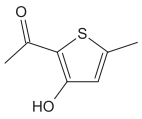
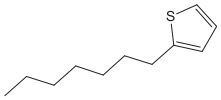
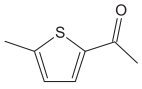
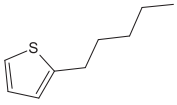
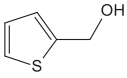
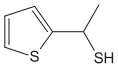
In addition to the S-oxidation, substituted thiophenes are also oxidized to an arene epoxide. Incubation of 2-phenylthiophene with either rat liver microsomes or CYP1A1 *in vitro* in the presence of NADPH formed two reactive intermediates, an S-oxide and an epoxide (Dansette et al., 2005). These intermediates in turn resulted in four types of products, as identified by ^{1}H NMR and MS (Fig. 3): a) dimers of 2-phenylthiophene S-oxide (2PTSOD), representing Diels-Alder type adducts; b) GSH adducts of the S-oxide metabolite, 2-phenylthiophene 2,5-dihydro-S-oxide (2PTGA), formed by 1,4-Michael-type addition of GSH to the ring; c) GSH adducts resulting from a nucleophilic attack of GSH on an intermediary metabolite that was predicted to be the 4,5-epoxide of 2-phenylthiophene (2PTGB); and d) 2-phenylthiolene thiolactone (2PTT), a rather unstable metabolite (Dansette et al., 2005). 2PTSOD and 2PTGA formation result from the S-oxide intermediate, 2PTGB forms from the epoxide intermediate and the 2PTT can be formed by oxygen isomerization in either pathway (Fig. 3).

Studies with thiophene pro-drugs, such as clopidogrel (Plavix®; (+)-(*S*)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)acetate) and prasugrel (Effient®; (*RS*)-5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate) have shown that the thiolactones formed after an initial oxidation of the thiophene ring (at position 2) are further oxidized resulting in ring opening and sulfenic acid isomeric metabolites (based on the position of the remaining double bond) (Dansette et al., 2009, 2010; Mansuy and Dansette, 2011). The first oxidation reaction is catalyzed by CYP 1A2, 2B6 and 2C19. The second oxidation and ring opening can be catalyzed by CYP 3A4, 2C9, 2B6 and 2C19 in the presence of NADPH leading to one isomer of sulfenic acid (α,β unsaturated; active metabolite), or by paraoxonase 1 (PON1) leading to the other isomer (inactive metabolite) of sulfenic acid (Dansette et al., 2009, 2012a,b; Gramec et al., 2014). Similar to PTT (Fig. 3), the thiolactones of these pro-drugs are formed by either S-oxidation or ring epoxidation (Dansette et al., 2009; Shimizu et al., 2009; Gramec et al., 2014).

5.2.3. The position of the acyl-substitution group: 2- and 3- isomers

The position of substitution on the thiophene ring plays a key

Table 2
Structural features of thiophene flavoring ingredients relevant to their metabolic fate.

2-Substitution		3-Substitution	
Alkyl-	Acyl-	Alkyl-	Acyl-
 3062[a]	 3209	 4184	 3-acetyl-2,5-dimethylthiophene
 3323	 4142	 4645	
 4137	 4643		
 4387			
 4642			
 4646			

[a] GRAS numbers.

role in the metabolic fate of thiophenes and subsequent reactivity toward cell components and downstream effects. Tienilic acid ([2,3-dichloro-4-(thiophene-2-carbonyl)phenoxy]acetic acid; TA) (a diuretic drug) and its regioisomer (TAI) differ only in the position of the aroyl substitution group (position 2 and 3, respectively) (Fig. 1). When these isomeric thiophenes were incubated *in vitro* with phenobarbital-treated rat liver microsomal fractions in the presence of NADPH and O_2, they resulted in different ratios of reactive metabolites (Dansette et al., 1990). Ring oxidation of ^{14}C-labelled TA was the predominant metabolic pathway, resulting primarily in formation of a 5-OH metabolite (65%), followed by a substantial fraction (30%) of other metabolic intermediates irreversibly bound onto microsomal proteins (described as "reactive fraction") and a relatively minor fraction (3%) of other polar metabolites (Dansette et al., 1990). In contrast, the 3-regioisomer, TAI, resulted predominantly in "reactive fraction" (80%) and lower yield of ring-hydroxylated product (18%) in the same system (Dansette et al., 1990). The reactivity of the intermediates of the 3-regioisomer was approximately 5-fold higher (covalent binding of 12 nmol/nmol CYP/20min) than that of the 2-keto isomer (2.5 nmol/nmol CYP/20min). The "reactive fraction" results from a metabolic pathway other than ring oxidation, as described below.

While ring oxidation leading to the 5-OH product is the main metabolic pathway for TA (Dansette et al., 1990), TAI is subject to S-oxidation (Mansuy et al., 1991; Valadon et al., 1996; Rademacher et al., 2012) as well as ring oxidation (Rademacher et al., 2012) (Fig. 4A and B). Oxidation of TAI by recombinant CYP 2C9 in the presence of NADPH, results in two products, an *S*-oxide dimer and a pair of hydroxythiophene/thiolactone tautomers (Fig. 4 B), from the

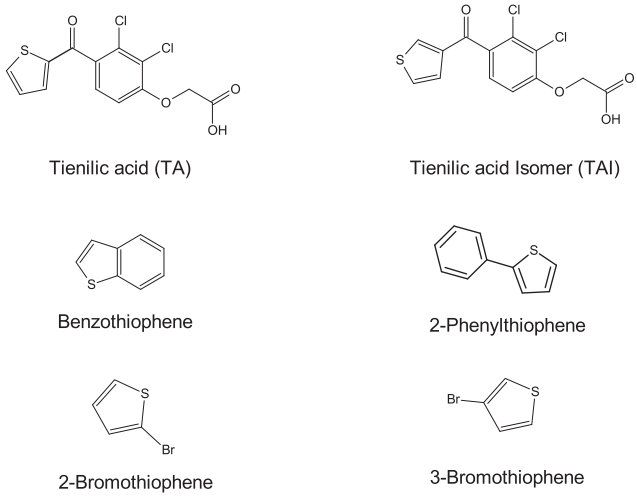


Fig. 1. Substituted model thiophene structures tested in metabolic studies.

S-oxide and arene epoxide intermediates, respectively (Rademacher et al., 2012). According to *in silico* modelling, 5-OH-TA and 5-OH-TAI (subsequently rearranged to their thiolactone isomers) result from an arene epoxide intermediate rather than from an *S*-oxide with subsequent 1–5 oxygen migration (Rademacher et al., 2012). Although TA *S*-oxide intermediate and its products were not detected by Rademacher and others, this study used Supersomes™ engineered to express CYP 2C9 and may

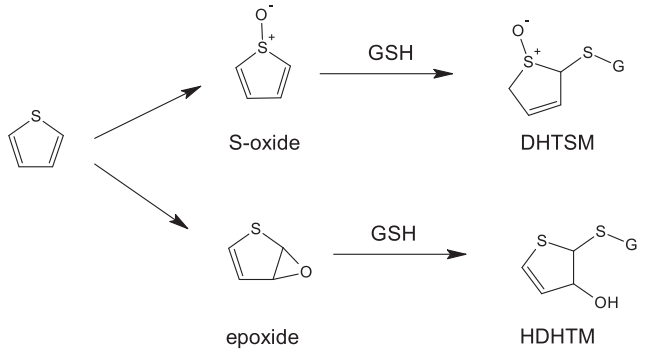


Fig. 2. Known metabolites of thiophene oxidation *via* sulfoxide and epoxide intermediates (Dansette et al., 1992). DHTSM: dihydrothiophene sulfoxide mercapturate; HDHTM: 3-hydroxy dihydrothiophene mercapturate.

microsomes *in vitro*: in the absence of a sulfur nucleophile, 57% soluble metabolites and 21% microsome-bound metabolites were detected; in the presence of 100 μM mercaptoethanol or N-acetylcysteine, the fraction of soluble metabolites increased to approximately 93% of parent compound (>60% consisted of the two diastereomers of the 2-mercapturate of TAI *S*-sulfoxide) and the microsome-bound species decreased to a mere 5%; with increasing concentrations (1–2 mM) of nucleophile, further metabolic transformations occur, including additional covalent binding of mercaptoethanol on position 4 of the ring and on the ring S, followed by ring opening (~57%), ring closure and almost exclusive formation of 4-mercaptoethanol conjugate of 4,5-dihydro-TAI as the final stable metabolite (Fig. 5). Nucleophile conjugation of activated thiophene intermediates has also been confirmed *in vivo* (see section below).

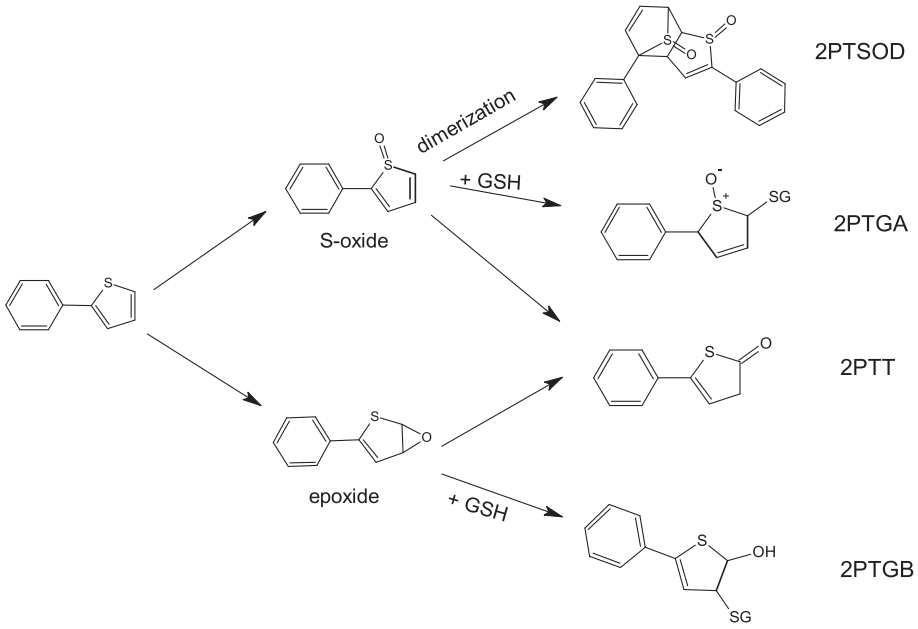


Fig. 3. Metabolism of 2-phenylthiophene (adapted from Dansette et al., 2005).

underrepresent other CYP isoforms (e.g., CYP1A1) previously shown to catalyze the S-oxidation of 2-phenylthiophene (Dansette et al., 2005).

5.3. Conjugation with sulfur nucleophiles

Sulfur nucleophiles are critical in quenching the reactive intermediates of substituted thiophenes, thus mitigating potential for toxicity from the intermediate. The fraction of stable water soluble metabolites relative to reactive metabolites bound on microsomal proteins increases in the presence of mercaptoethanol, *N*-acetylcysteine or GSH *in vitro* (Dansette et al., 1990; Valadon et al., 1996). Empirically, the presence of GSH or other sulfur nucleophiles favored the formation of the more stable 5-OH metabolite for both TA and TAI, relative to the reactive metabolites involved in microsomal binding, which were reduced up to 10-fold (Dansette et al., 1990). This shift in reactivity presumably results from the sequestration of *S*-oxide derived reactive intermediates by GSH as shown in Fig. 5. The available concentration of sulfur nucleophiles is directly related to the degree of quenching of the reactive intermediates resulting from incubation of TAI with rat liver

5.3.1. Factors determining conjugation efficiency

Both the type of CYP isoforms and the presence or absence of GSH determines the relative yield of reactive intermediates and the final metabolic products. As expected, the GSH conjugates of 2-phenylthiophene (2PTGA and 2PTGB; Fig. 3) are formed at higher proportions (20% and 30%, respectively) in the presence of GSH following activation by liver microsomes at the expense of both 2PTSOD and PTT (reduced from 35% to 12% and from 30% to 3%, respectively) (Dansette et al., 2005). The *S*-oxide dimer of 2-phenylthiophene is the primary product (31%) of recombinant CYP 1A1 in the presence of GSH, followed by PTT and the GSH conjugates A and B (4%, 6% and 3%, respectively). This metabolite profile indicates that CYP 1A1 is not involved in the formation of an arene epoxide and that other CYP enzymes present in microsomal preparations are involved in the formation of both *S*-oxide and epoxide pathways (Dansette et al., 2005). At least one other CYP enzyme involved is the CYP 2C9 (Rademacher et al., 2012). This enzyme oxidizes both the 2- and 3-aroyl thiophene isomers and catalyzes both the S-oxidation and arene oxidation pathways (Rademacher et al., 2012).

The location of GSH conjugation of activated thiophene

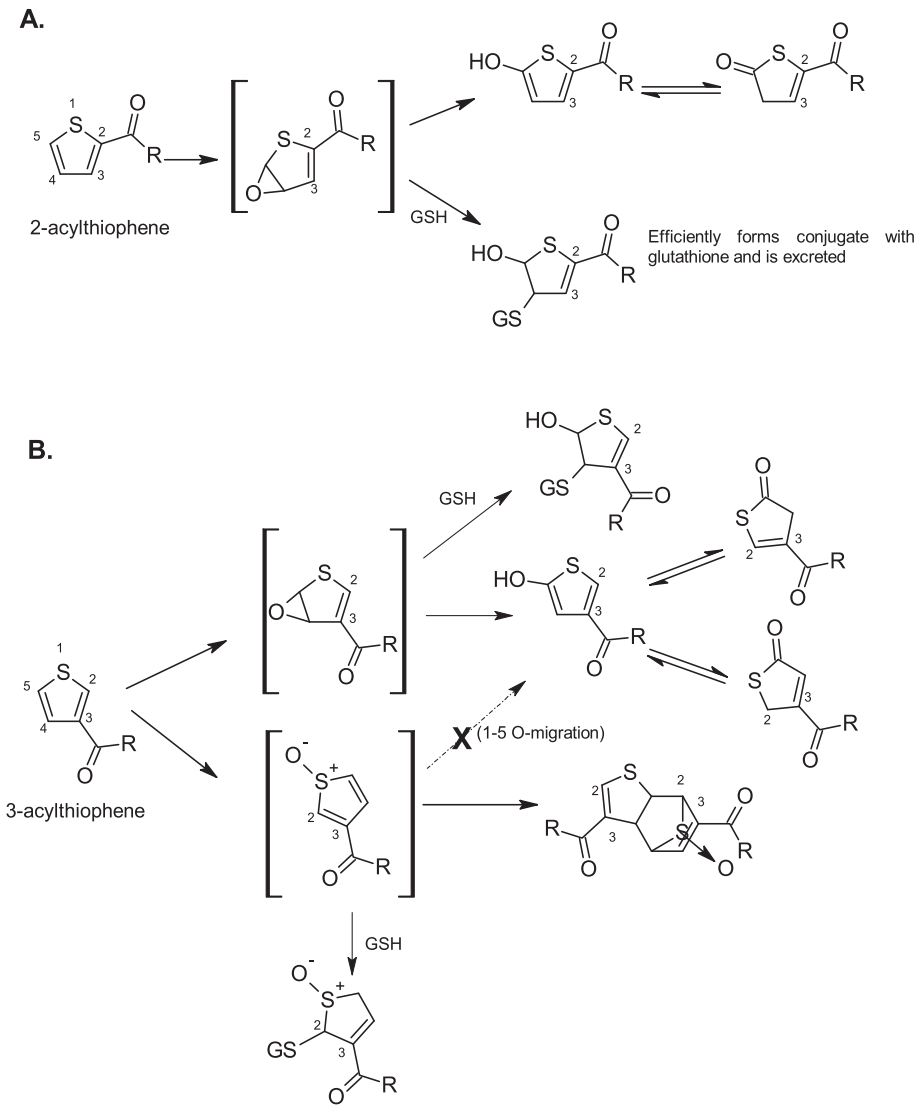


Fig. 4. Possible metabolic pathways for the biotransformation of 2-acylthiophene (A) compared to 3-acylthiophene (B), based on documented empirical evidence and *in silico* modelling (Dansette et al., 2005, Valadon et al., 1996; Rademacher et al., 2012).

intermediates depends on the mechanism of metabolic activation (S-oxidation or ring oxidation) and on the presence of other substituents. For example, GSH binding following *S*-oxidation occurs *via* Michael-type reaction on a carbon adjacent to the ring sulfur (ring positions 2, 5) (Dansette et al., 1992, 2005; Valadon et al., 1996). Binding of GSH on the ring occurs at position 3 following ring epoxidation (Dansette et al., 1992; 2005). In 2-substituted thiophenes such as 2-phenylthiophene (2 PT) (Dansette et al., 2005) or 2-acetylthiophene (Yan et al., 2005), GSH binding occurs at the ring position not bearing the substituent (position 5).

5.4. *In vivo metabolism*

The metabolic fate of thiophenes *in vivo* is consistent with the pathways described above, based on the nature of urinary metabolites detected following oral administration in Sprague-Dawley rats (Valadon et al., 1996). Nucleophilic conjugation of activated thiophene intermediates is also confirmed *in vivo* (Valadon et al., 1996). GSH, present at high concentrations in hepatocytes (1–5 mM), is the main sulfur nucleophile *in vivo* resulting in formation of mercapturate conjugates similar to those seen *in vitro* with mercaptoethanol. Injection (*i.p.*) of male Sprague-Dawley rats with 30 mg/kg bw of ^{14}C-labelled TAI (at the keto position), resulted in a mixture of two diastereomers of the 4-mercapturate conjugate of 4,5-dihydro-TAI (Valadon et al., 1996). However, unlike the yields of mercapturate conjugates seen *in vitro*, recovery of metabolites in the urine does not fully account for the administered dose. Only 20% of the administered dose of radiolabelled TAI was accounted for in the urine within 24 h and the urinary mercapturate conjugates accounted for only 2% of the administered radioactivity and together for approximately 15% of all urinary radioactivity. Relatively higher fractions of mercapturate conjugates (40% and 30%) were reported in other studies (Bray et al., 1971; Dansette et al., 1992) in male Sprague-Dawley rats. In a limited study, urinary mercapturate conjugates in rats were reported for non-substituted thiophene and its 2-bromo- and 3-bromo-derivatives (3%, 6% and 14%, respectively) (Hickman et al., 1992). Formation of thiophene sulfoxide was not reported in this study for the bromo-thiophene derivatives (Hickman et al., 1992). The reasons for the low recoveries of mercapturate and other urinary metabolites in these

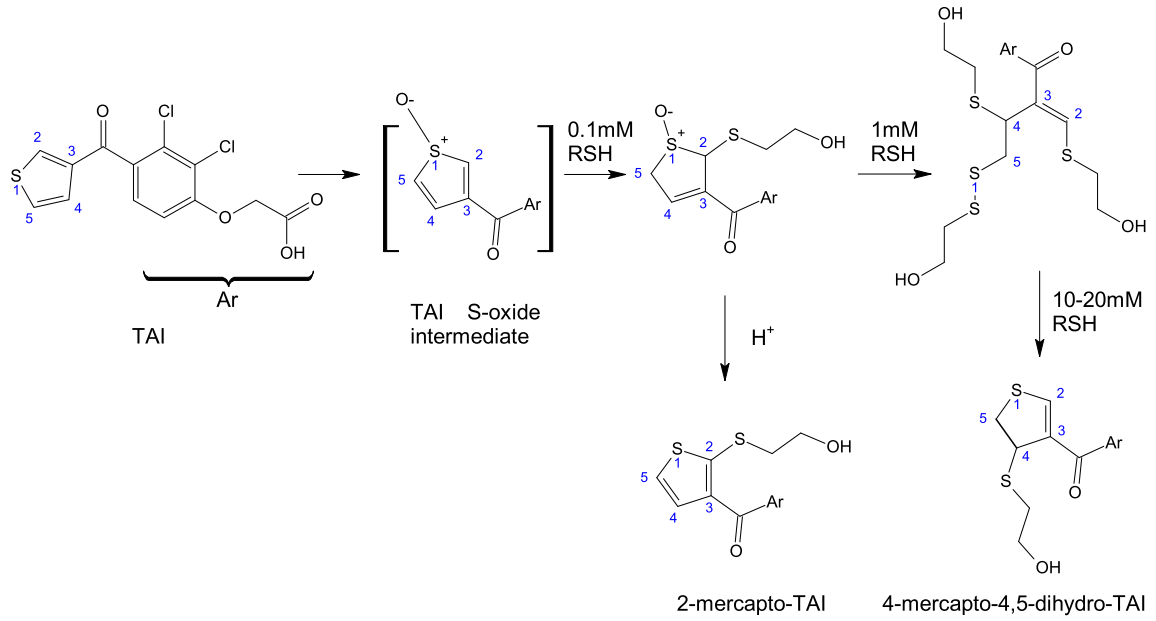


Fig. 5. Nucleophile conjugates of TAI sulfoxides detected *in vitro* in the presence of liver microsomes, NADPH and mercaptoethanol, at increasing concentrations of nucleophile. The 2-mercapto-TAI results only under acidic conditions (Valadon et al., 1996).

studies are unclear and it should be noted that no other tissues were examined that might have clarified the alternate fate of the administered thiophene compounds.

In humans, metabolism of thiophene pro-drugs, such as clopidogrel and ticlopidine results in the formation of a thiolactone (e.g. 2-oxo-clopidogrel) followed by oxidation of the ring to the sulfenic acid that is subsequently conjugated with GSH (Gramec et al., 2014; Farid et al., 2010). In prasugrel, the thiolactone is formed *via* isomerization of the 2-hydroxyl group that results from ester hydrolysis and it also proceeds to sulfenic acid metabolites (Farid et al., 2010). Oxidation of the parent compounds to the thiolactones is mediated *via* CYP 1A2, 2B6 and 2C19, whereas the second oxidation step is mediated *via* CYP 3A4, 2C9, 2B6 and 2C19 (Kazui et al., 2010). Urinary excretion of ticlopidine and prasugrel accounts for 60% and 68%, respectively, of the administered dose of these substances. The major urinary metabolite of prasugrel (21% of administered dose) is derived from a hydroxylated thiophene intermediate while other products are derived from other hydrolysis metabolites (Farid et al., 2010). Approximately 20–27% of the administered dose is recovered in the feces either from biliary excretion or as non-absorbed compound (Farid et al., 2010).

5.5. Summary of metabolism

From the studies described above, the key structural features that determine the metabolic fate of model substituted thiophene compounds were characterized, including a) the presence and number of substitution groups, b) the type of substituents, e.g. alkyl or acyl side chains, and c) their location on the ring (Rademacher et al., 2012; Gramec et al., 2014). Generally, a) metabolites derived from *S*-oxide intermediates are more reactive than those of the arene oxide intermediates (Dansette et al., 1990), b) metabolites of *S*-oxides of acyl-substituted thiophenes are more reactive than the equivalent of thiophenes lacking the acyl substituent (Mansuy et al., 1991) and c) 3-acyl substituted thiophenes favor *S*-oxide intermediates more than 2-acyl isomers (Dansette et al., 1990; Rademacher et al., 2012).

6. Toxicology

6.1. Acute toxicity

A limited number of studies have assessed the acute oral toxicity of thiophenes and indicate a low toxic potential. In a GLP oral acute toxicity study in Sprague-Dawley rats, 3-acetyl-2,5-dimethylthiophene had an LD_{50} of 455 mg/kg bw/day in males and 594 mg/kg bw/day in females (combined LD_{50} of 510 mg/kg bw/day for male and female) (Mallory, 1982). 2-Thienyldisulfide had an oral LD_{50} value of 400 mg/kg bw in male and female mice (Table 3) (Moran and Easterday, 1980). The structurally related thiophene-2-carboxaldehyde had an oral LD_{50} of 565 mg/kg bw in mice and 915 mg/kg bw in rats (Sharp, 1979). The unsubstituted thiophene has reported LD_{50} values of 1902 and > 500 mg/kg bw in mice (O'Donoghue, 1979; Eli Lilly Co, 1992) and 1131 and 3120 mg/kg bw in rats (O'Donoghue, 1979; Younger, 1965). An acute neurotoxicity and behavioral toxicity study was conducted in Wistar-Kyoto rats with unsubstituted thiophene, based on earlier studies indicating that thiophene induced degeneration of granule cells selectively in the cerebellum in rats. Treatment with daily intramuscular injections of 0.15 mL thiophene (equivalent to 651–814 mg/kg bw/day)[1] for 3 days resulted in ataxia and convulsions in all animals within 24 h (Mori et al., 2000).

6.2. Short-term toxicity studies

6.2.1. 5-Methyl-2-thiophenecarboxaldehyde and 3-acetyl-2,5-dimethylthiophene

5-Methyl-2-thiophenecarboxaldehyde (FEMA No. 3209) and 3-acetyl-2,5-dimethylthiophene were tested in male and female Fischer 344 rats (5/sex/group) for 14 days, each at a single dietary concentration estimated to give an average daily intake of 10 mg/kg bw (Gill and Van Miller, 1987). Neither substance caused any notable or biologically relevant toxicity. No treatment-related

[1] Density = 1.086 g/mL; for rats weighing 200-250 g.

Table 3
Acute oral toxicity studies for thiophene derivatives used as flavoring ingredients.

Flavoring ingredient	Species, Sex[a]	LD_{50} (mg/kg bw)	Reference
2-Thienyldisulfide	Mouse; M,F	400	Moran and Easterday 1980
3-Acetyl-2,5-dimethylthiophene	Rat; M,F	455 (M) 594 (F)	Mallory 1982

[a] M = Male; F=Female; NR=Not Reported.

lesions were found from macroscopic and histopathological examinations compared to control (Gill and Van Miller, 1987).

6.2.2. 5-Ethylthiophene-2-carboxaldehyde

This compound is not a flavoring ingredient but it is structurally directly related to the flavoring 5-methyl-2-thiophenecarboxaldehyde (FEMA No 3209). In a 14-day dietary study, male and female Hsd:SD® rats (3/sex/group) were fed a diet containing 0, 240, 2400 and 12,000 ppm of 5-ethylthiophene-2-carboxaldehyde, estimated to correspond to 22, 194 and 676 mg/kg bw/day for males and 22, 195 and 686 mg/kg bw/day for females (Bauter, 2012). No mortality was observed throughout the course of the study. Clinical signs in both males and females included reductions in fecal volume, body weight gain, body weight (up to 21%), food consumption and food efficiency, that were statistically significant at 12,000 ppm for all males but not females. The males in the 2400 ppm group also showed a small reduction in body weight in the first 3 days (2.2%). Other observations in males of the high dose group included red oral/nasal discharge, red facial and anogenital staining, nose/snout swelling, cuts and eschar, tremors, biting and hyperactivity. This study provided an upper tolerated intake level of 240 ppm for male rats and 2400 ppm for females (equivalent to 22 and 195 mg/kg bw/day, respectively) for the follow-up study of longer duration (described below) (Bauter, 2012).

6.3. Subchronic toxicity studies

The results of subchronic toxicity studies are summarized in Table 4.

6.3.1. 5-Ethylthiophene-2-carboxaldehyde

As stated above, this compound is not a FEMA GRAS flavoring ingredient but it is used in other global regions as a flavoring substance (EFSA, 2015), and is structurally related to the flavoring 5-methyl-2-thiophenecarboxaldehyde (FEMA No 3209). It was administered to male and female Sprague-Dawley® Crl:CD IGS rats (10/sex/group), in an OECD compliant 90-day study, at doses of 0, 2, 7.5 and 75 mg/kg bw/day by gavage (Bauter, 2013a). Since the liver was a target organ, complete histopathological examinations of liver tissue were conducted and other tissues were preserved for future histopathological examination.

There were no unscheduled deaths in the test groups. There were no remarkable findings in ophthalmic examinations, urine analysis, clinical pathology, macroscopic, microscopic, or organ weight changes and no other changes in hematology, coagulation or serum chemistry parameters that were considered biologically relevant. Upon microscopic examination, high-dose males and females showed atrophy of the submandibular salivary gland but there were no comparable findings at the two lower doses. Based on this 90-day oral toxicity study in rats, the no-observed-adverse-effect level (NOAEL) for 5-ethylthiophene-2-carboxaldehyde administered *via* gavage was 7.5 mg/kg bw/day for males and females (Bauter, 2013a). Based on structural similarity, the Panel considered this NOAEL appropriate for 5-methyl-2-thiophenecarboxaldehyde (flavoring ingredient). Compared to the estimated human exposure of the flavoring ingredient (MSDI; 0.002 μg/kg bw/day), this NOAEL results in a margin of safety of 3,750,000 (Table 4).

6.3.2. 2-Thienyldisulfide

A 90-day dietary study for 2-thienyldisulfide (FEMA No 3323) was conducted in weanling rats (15/sex/group strain not reported) at a single target intake level of 0.29 mg/kg bw/day (Morgareidge and Oser, 1970). This level was designed to be 100 times higher than the estimated Possible Average Daily Intake (PADI) from its use as a flavoring substance at the time of the study. The PADI method (Morgareidge and Oser, 1970) assumes that all foods in a food category always contain that substance and that the food category is consumed daily (Oser and Hall, 1977), and therefore, it is a very conservative overestimate of the average human daily intake for low volume flavoring substances (Hall and Ford, 1999).

Neither overt signs of toxicity nor mortality were noted between 2-thienyldisulfide treated and control animals. There were no differences in body weights and food consumption, hematology, blood chemistry and urine analysis, absolute or relative liver and kidney weights and no evidence of gross pathology or histopathology of major organs. The dose of 0.29 mg/kg bw/day, the only dose tested, would result in a NOAEL giving a margin of safety of 290,000 compared to the estimated human dietary exposure (MSDI, 0.001 μg/kg bw/day) to this substance from its intended use as a flavoring ingredient (Table 4).

6.3.3. 2-Pentylthiophene

2-Pentylthiopene (FEMA No 4387) was administered to Sprague-Dawley Crl:CD® (SD) IGS BR rats (5/sex/group) at dose levels of 0, 15, 150 or 500 mg/kg bw/day by gavage, in a 28-day

Table 4
Subchronic toxicity studies for thiophene derivatives used as flavoring ingredients.

Flavoring ingredient	Species, Sex[a]	No. Test groups/Group size[b]	Route	Duration (days)	NOAEL (mg/kg bw/d)	Margin of Safety[c]	Reference
2-Thienyldisulfide	Rats; M,F	1/30	Dietary	90	0.29[d]	290,000	Morgareidge and Oser 1970
2-Pentylthiophene	Rats; M,F	3/10	Gavage	28	15	75,000,000	Dhinsa et al., 2006
2-Pentylthiophene	Rats; M,F	1/10	Gavage	28	3.0[d]	15,000,000	Marr and Watson, 2007
2-Pentylthiophene	Rats; M,F	3/20	Dietary	90	33	165,000,000	Bauter, 2013b
5-Ethylthiophene-2-carboxaldehyde[e]	Rats; M.F	3/20	Gavage	90	7.5	3,750,000	Bauter, 2013a

[a] M = Male; F=Female.
[b] Total number of test groups does not include control animals. Total number per test group includes both male and female animals.
[c] Assuming intake as high as the combined intake of the group (Sum of MSDI = 0.003 μg/kg bw/day).
[d] The study was performed at a single dose level and produced no adverse effects. The NOAEL is probably higher than the dose level reported here.
[e] NOAEL of structural analogue applies to the flavoring substance 5-methyl-2-thiophenecarboxaldehyde.

study (Dhinsa et al., 2006). Toxicity was evident in all animals at the highest dose (500 mg/kg bw/day) and most animals in the middle dose. Minor non-adverse effects were seen in only two males at the lowest dose.

More specifically, adverse effects observed in both males and females at the middle and high doses (150 and 500 mg/kg bw/day) included hemolytic anemia, increases in bilirubin and alanine aminotransferase, increases in absolute and relative liver, kidney and spleen weights, centrilobular hepatocyte hypertrophy and globular accumulations of eosinophilic material in the renal tubules. Splenic toxicity included increased incidence of higher grades of severity of extramedullary hematopoiesis, hemosiderin accumulation and associated hyperemia. The centrilobular hepatocyte hypertrophy is likely related to constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR) activation, and consequently, CYP induction (Williams and Iatropoulos, 2002; Graham and Lake, 2008; Thoolen et al., 2010; Hall et al., 2012). Hypertrophy of follicle-lining cells of the thyroid gland in males was observed only at 150 mg/kg bw/day and it was postulated to be secondary to activation of CAR/PXR in the liver, leading to increased metabolism of circulating thyroid hormones and compensatory increased thyroid stimulating hormone. This mechanism is considered unique to rodents and thus not relevant to humans (Graham and Lake, 2008). At the highest dose (500 mg/kg bw/day), hemosiderin pigment deposits in the liver and in the kidney tubular epithelium, renal tubular hypertrophy and urinary bladder epithelial hyperplasia with associated inflammatory features in both tissues, were found. With the exception of increased salivation in two animals and a slight increase in blood bilirubin, no treatment-related adverse effects were detected at 15 mg/kg bw/day, providing a NOAEL as reported by the authors of 15 mg/kg bw/day (Dhinsa et al., 2006) that results in a margin of safety of 75,000,000 compared to the estimated exposure (MSDI, 0.0002 μg/kg bw/day) to this flavoring substance (Table 4).

The slight increase in blood bilirubin at the lowest dose (15 mg/kg bw/day) was assessed in a follow-up 28-day study to evaluate effects of 2-pentylthiophene in the spleen (Marr and Watson, 2007). The substance was administered at a single dose level of 3 mg/kg bw/day by gavage to Sprague-Dawley Crl:CD® (SD) IGS BR rats (5/sex). No treatment-related changes were noted in clinical pathology, body weight growth, hematology and blood chemistry, nor were any significant other toxicological effects in spleen weight or macroscopic abnormalities (histopathological examination was not conducted in this study). Based on this, a NOAEL of 3 mg/kg bw/day, the only dose tested, could be established and results in a margin of safety greater than 15,000,000 compared to the estimated combined human exposure (MSDI, 0.0002 μg/kg bw/day) of flavor substances in this group (Table 4).

In a 90-day dietary study, CRL Sprague-Dawley CD®IGS rats (10/sex/group) were fed a diet containing 0, 28, 140 and 700 ppm of 2-pentylthiophene (Bauter, 2013b). The dose was determined to be 1.4, 7 and 33 mg/kg bw per day for males and 1.5, 8 and 39 mg/kg bw per day for females, following stability analysis of the substance. No further analysis to explain the reduction of stability in the diet was performed. No mortality and no significant clinical signs of toxicity were observed among hematology, clinical chemistry, coagulation and urine analysis parameters, or body weight changes. Increased relative kidney weight seen in females of the high dose group was the only macroscopic finding, and it was not toxicologically significant in the absence of associated specific gross or microscopic findings. No microscopic observations were detected in the any of the tissues examined. In addition, there was no increase in blood bilirubin at any dose level in this study. Therefore, the NOAEL for 2-pentythiophene in the diet was determined to be 33 and 39 mg/kg bw/day for males and females, respectively (Bauter, 2013b) and results in a margin of safety of 165,000,000 compared to its estimated exposure (MSDI, 0.0002 μg/kg bw/day) to this flavoring substance (Table 4).

6.3.4. Thiophene

Unsubstituted thiophene is a substance that is approved for use as a flavor ingredient in Europe but does not have FEMA GRAS status for the United States. In a 42-day repeat oral dosing study, thiophene was administered to male and female Sprague-Dawley rats (13/sex/group) *via* gavage, at doses of 0, 25, 100, or 400 mg/kg bw/day (Nagao, 2006). Loss of balance was seen immediately after administration of 100 or 400 mg/kg bw/day of thiophene. Adverse effects were observed at 100 and 400 mg/kg bw/day compared to control groups, including blood chemistry and organ weights and histopathological changes in the liver, kidney, spleen and cerebellum that increased in severity at the high dose. No adverse effects were noted at the lowest dose level in this study, which would indicate a NOAEL of 25 mg/kg bw/day (Nagao, 2006).

6.4. Developmental toxicity studies

6.4.1. Thiophene

Reproductive and developmental toxicity endpoints were evaluated as part of a short term toxicity study (Nagao, 2006). Sprague-Dawley rats (13/sex/group) were administered 0, 25, 100, or 400 mg/kg bw/day of thiophene by gavage, for a 42-day period starting 14 days prior to cohabitation and extending through day 4 of lactation. There were no adverse effects on any of the reproductive endpoints evaluated, including copulation, ovulation, or fertility in any of the treatment groups compared to control groups. Dams treated with 100 or 400 mg/kg bw/day of thiophene showed histopathological changes in the cerebellum (pyknosis and necrosis of granular cells) and abnormal lactation. No morphological abnormalities were found in the offspring, with the exception of reduced birth weights and decreased body weights and viability at postnatal day 4 in pups born to dams treated with 400 mg/kg bw/day. The no-observed-effect-level (NOEL) for reproductive toxicity was reported by the authors to be 25 mg/kg bw/day for female rats and 400 mg/kg bw/day for males (Nagao, 2006). The NOEL for developmental toxicity reported by the authors was 100 mg/kg bw/day.

6.5. Chronic toxicity studies

No long-term toxicity studies or carcinogenicity studies were found in the public literature for any of the eight flavoring substances in this reevaluation, and no structural analogues were found that have been tested in chronic rodent cancer bioassays.

6.6. Genotoxicity

In vitro and *in vivo* genotoxicity studies were recently conducted for 5-methyl-2-thiophenecarboxaldehyde and 3-acetyl-2,5-dimethylthiophene according to current OECD guidelines and GLP standards. The results of these and other studies are summarized in Tables 5 and 6 and are described below.

6.6.1. 5-Methyl-2-thiophenecarboxaldehyde

6.6.1.1. In vitro. The mutagenicity of 5-methyl-2-thiophenecarboxaldehyde was tested according to OECD guidelines in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102, both in the absence and in the presence of a metabolic activation system (S-9) from livers of rats induced with Aroclor 1254, in three experiments (Beevers, 2009). No dose-related, reproducible or statistically significant increases in revertant numbers were observed in any of the test strains, at

Table 5
In vitro genotoxicity studies for thiophene derivatives used as flavoring ingredients.

Substance	Assay	Test system	Concentration	Results	Reference
5-Methyl-2-thiophenecarboxaldehyde	Reverse mutation	*Salmonella typhimurium* TA98, TA100	4-100 μmole/plate	Negative[a]	Lee et al., 1994
5-Methyl-2-thiophenecarboxaldehyde	Reverse mutation	*S. typhimurium* TA98, TA1535, TA1537 and TA102	0.32–1000 μg/plate[b]	Negative[a]	Beevers 2009
5-Methyl-2-thiophenecarboxaldehyde	Reverse mutation	*S. typhimurium* TA98 TA100, TA102, TA1535, and TA1537	10.24–1000 μg/plate[b, c, d] 10.24–1000 μg/plate[e, f, g] 25.6–2500 μg/plate[b, c, h] 4.096–400 μg/plate[e, f, i]	Negative[a]	Beevers 2009
5-Methyl-2-thiophenecarboxaldehyde	Reverse mutation	*S. typhimurium* TA100, TA1535, TA1537	156.25–5000 μg/plate[b, f, j] 156.25–5000 μg/plate[b, c, k]	Negative[a]	Beevers 2009
5-Methyl-2-thiophenecarboxaldehyde	Micronucleus Induction	Human peripheral blood lymphocytes (Female and Male Donors)	600-1000 μg/ml[c, l] 50-70 μg/mL[f, l] 120-350 μg/mL[c, m] 50-80 μg/mL[f, l]	Negative[c] Weak Positive[e]	Lloyd 2011
3-Acetyl-2,5-dimethylthiophene	Reverse mutation	*S. typhimurium* TA98 TA100, TA102, TA1535, and TA1537	1.6–5000 μg/plate[a]	Negative[a] Positive[f, n]	Lillford, 2009
3-Acetyl-2,5-dimethylthiophene	Reverse mutation	*S. typhimurium* TA98 TA100, TA102, TA1535, and TA1537	51.2–5000[a, b]	Negative[c] Positive[f, n]	Lillford, 2009

[a] In the absence and presence of metabolic activating system, S-9.
[b] Plate incorporation method.
[c] Without metabolic activation system, S-9.
[d] In strains TA98, TA102, TA1535 and TA1537.
[e] Pre-incubation method.
[f] With metabolic activation system, S-9.
[g] In strains TA98 and TA1535.
[h] In strains TA1535 and TA1537.
[i] In strains TA100, TA102 andTA1535.
[j] In strains TA100 and TA1535.
[k] In strains TA100, TA1535 and TA1537.
[l] 3 h incubation with 21 h recovery.
[m] 24 h incubation with no recovery.
[n] In strains TA98, TA100 and TA102.

Table 6
In vivo genotoxicity studies for thiophene derivatives used as flavoring ingredients.

Substance	Assay	Test system	Route of Administration	Doses and test conditions	Results	Reference
5-Methyl-2-Thiophenecarboxaldehyde	Micronucleus Assay	Rat bone marrow	Gavage	70, 350, and 700 mg/kg bw/day (males only)	Negative	Beevers, 2012
5-Methyl-2-Thiophenecarboxaldehyde	Comet assay	Rat liver	Gavage	70, 350, and 700 mg/kg bw/day (males only)	Negative	Beevers, 2012
3-Acetyl-2,5-dimethylthiophene	Micronucleus Assay	Muta™Mice (bone marrow)	Gavage, 28 days	125, 235, and 300 mg/kg bw/day (males only)	Negative	Beevers, 2013
3-Acetyl-2,5-dimethylthiophene	Induction of *lacZ*⁻ mutations	Muta™Mice (liver and duodenum)	Gavage, 28 days	125, 235, and 300 mg/kg bw/day (males only)	Negative (duodenum) Positive (liver)	Beevers, 2013

concentrations up to either the limit of toxicity or 5000 μg/plate (the maximum recommended concentration according to current regulatory guidelines).

In an OECD-compliant *in vitro* micronucleus assay with cultured human peripheral blood lymphocytes, 5-methyl-2-thiophenecarboxaldehyde was tested for 3 h followed by 21 h recovery (3 + 21 h) in the absence and presence of S-9 from livers of rats induced with Aroclor 1254, or for 24 h without recovery (24 + 0 h) in the absence of S-9 (Lloyd, 2011). In the 3 + 21 h in the presence of S-9, cultures treated with 50, 60 and 70 μg/mL of 5-methyl-2-thiophenecarboxaldehyde showed a weak positive response, with only single replicate cultures exceeding the normal historical control range. In the absence of S-9, no biologically relevant increase in the MNBN frequencies were found in cells treated for 3 + 21 h (600, 900 and 1000 μg/mL), or for 24 + 0 h (120, 240, 300, and 350 μg/mL). Despite the statistically significant differences, all mean MNBN frequencies of vehicle control and treated cultures were within the normal range and the results were considered equivocal.

6.6.1.2. In vivo. The equivocal evidence of genotoxicity observed in the *in vitro* micronucleus test was further examined in an OECD-compliant *in vivo* study which included a Comet assay in the liver, the primary site of metabolism, and the micronucleus assay in the bone marrow in rats (Beevers, 2012). Han-Wistar male rats (n = 6/group) were treated with 5-methyl-2-thiophenecarboxaldehyde by oral gavage for three consecutive days and at doses of 70, 350 and 700 mg/kg bw/day (ethyl

methanesulfonate, 150 mg/kg bw/day was used as a positive control). The highest dose used was determined to be the maximum tolerated dose (MTD) in a preliminary range finding study, where mortality was observed at 1000 mg/kg bw/day. Treatment did not cause any overt toxicity, with the exceptions of increased levels of serum aspartate aminotransferase at 700 mg/kg bw/day and a dose-dependent decrease in total cholesterol levels. Liver exposure to the substance was indicated by histological observation of glycogen deposits at 350 and 700 mg/kg bw/day along with changes in liver enzymes. However, 5-methyl-2-thiophenecarboxaldehyde did not induce DNA damage in the liver; although mean Comet tail intensities and tail moments were slightly higher in treated compared to control animals (statistical analysis was not performed), only isolated replicates exceeded the historical control range. Since most values for these parameters of DNA migration were below the laboratory's historical control range the results were considered to fall within the normal level of variation. No cytotoxicity, necrosis or apoptosis were observed in cell suspensions.

Similarly, no statistically significant increases in micronucleus frequency were detected, indicating no evidence of genotoxicity in the bone marrow in this OECD compliant study. Although there was no bone marrow toxicity, exposure was verified based on liver effects as described above. The Panel concluded that 5-methyl-2-thiophenecarboxaldehyde was not genotoxic *in vivo* following oral intake up to the MTD of 700 mg/kg bw/day.

6.6.2. 3-Acetyl-2,5-dimethylthiophene

6.6.2.1. In vitro. The mutagenicity of 3-acetyl-2,5-dimethylthiophene was tested in an Ames assay in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102, both in the absence and in the presence of metabolic activation (S-9 from livers of rats induced with Aroclor 1254), at concentrations up to 5000 μg/plate using the plate incorporation methodology (Lillford, 2009). Toxicity was observed in all strains at 2000 and 5000 μg/plate both in the absence and presence of S-9. In the presence of S-9, clear, dose-related, statistically significant ($p < 0.01$) and reproducible increases in revertant numbers (up to 14-fold) were observed in test strains TA98 and TA100, and to a much lower extent (maximum 1.4-fold) in strain TA102 (but not in strains TA1535 and TA1537). In contrast, no mutagenic activity was observed in the absence of S-9 in any strain. These results indicate that the mutagenic activity is related to metabolic products of 3-acetyl-2,5-dimethylthiophene.

6.6.2.2. In vivo. The Muta™Mouse (*lacZ/GalE*) assay with an *in vivo* micronucleus component is considered an appropriate follow-up when positive results are obtained in the *in vitro* bacterial reverse mutation assay (EFSA, 2011; OECD, 2011). Conclusions from the study results are made by the laboratory performing the study and evaluated by the Expert Panel.

The Muta™Mouse (*lacZ/galE*) assay detects the induction of point mutations and small deletions in the *lacZ* gene, in any target tissue where a substance is active. Briefly, a λ GT10 vector containing a bacterial *lacZ* gene is incorporated in the genome of the Muta™Mouse CD_2-lacZ80/HazfBR strain and is therefore present in every nucleated cell of the animal. Following treatment of Muta™Mice with a test substance, cellular DNA is extracted from tissues of interest and packaged into λ bacteriophage which is then used to transfect *E. coli* C *lac*$^-$ *galE*$^-$ Kan^r (or *galE*$^-$ Amp^r) cultures. The number of plaques produced on non-selection (titer) plates indicates the total number of viable bacteriophage units (transfected *E. coli*). Mutations in the *lacZ* gene result in bacterial colonies resistant to phenyl-galactose (P-Gal), a galactosidase substrate that is toxic to *gal*E$^-$ bacterial strains expressing the non-mutated wild type *lacZ* gene. Bacterial colonies of non-transfected *E. coli* are also viable on plates containing P-Gal medium. However, only the bacteria transfected with the bacteriophage carrying the mutated *lacZ* gene result in bacterial lysis upon bacteriophage replication and produce visible plaques, on a lawn of non-transfected (but viable) bacteria. Mutant frequency is calculated as the ratio of the number of bacteriophage containing mutated *lacZ* transgenes (i.e., plaques on positive selection plates) over the total number of viable bacteriophage (i.e., visible plaques on titer plates) (Lambert et al., 2005).

Therefore, the Muta™Mouse assay was selected to further probe the mutagenic potential of 3-acetyl-2,5-dimethylthiophene seen *in vitro*. The liver and the duodenum were chosen as the most appropriate tissues to address the potential for mutation, representing the site of most significant metabolism and the site of first contact, respectively. Micronuclei were measured in peripheral blood reticulocytes. The MTD of 350 mg/kg bw was determined in a dose-range finding experiment ($n = 3$/sex/dose) following 7 days of dosing by oral gavage, based on the toxicity displayed at higher doses in this experiment.

Male Muta™Mouse CD_2-lacZ80/HazfBR mice (6/dose) were treated daily for 28 days by oral gavage with 3-acetyl-2,5-dimethylthiophene at doses of 0, 120, 235 and 350 mg/kg bw/day (the top dose was lowered to 300 mg/kg bw/day after 2 days of dosing due to signs of distress). Animals were sacrificed three days after the final administration (day 31) and subjected to necropsy. The functionality and validity of the mutation assays were ensured by including a tissue-matched positive control DNA (from ethyl nitrosourea-treated animals).

Treatment of Muta™Mice with 3-acetyl-2,5-dimethylthiophene caused a dose-dependent increase in the mutation frequency of the *lacZ* transgene in the liver, which was statistically significant in the middle and high dose groups and exceeded both concurrent and historical control means. A small increase in *lac*Z mutation frequency was also noted in the duodenum in the low and middle dose groups, but it was not statistically significant and remained within the historical control range.

In contrast, no dose- or time-related increases in the frequency of micronucleated peripheral blood reticulocytes were seen in these animals. Small and statistically significant but isolated increases in micronuclei (at the high dose on day 4 and the middle dose on day 31) were considered to be within the inherent variation of the assay and without biological significance. Considering the positive mutagenic activity in the liver but not in the duodenum or in the micronucleus assay, the mutagenic potential *in vivo* appears to be related to a liver metabolite of 3-acetyl-2,5-dimethylthiophene (Beevers, 2013). These results are consistent with the pattern of mutagenicity seen *in vitro*.

Previously available genotoxicity data on structurally related substances provide additional context for the interpretation of the results of the genotoxicity studies for these two materials. A summary of genotoxicity studies on other thiophene compounds is presented below:

6.6.3. Earlier genotoxicity studies with thiophene derivatives

6.6.3.1. Thiophenes negative for mutagenicity

6.6.3.1.1. Unsubstituted thiophene. Unsubstituted thiophene was negative for mutagenicity in the Ames assay with *Salmonella typhimurium* strains TA100, TA1535, TA1537, TA97 and TA98 in several studies, at concentrations up to 10,000 μg/plate with and without metabolic activation from rat or hamster liver preparations (Zeiger et al., 1987a,b; Shibuya, 2006; Aeschbacher et al., 1989). Similarly, there was no mutagenic activity observed in a reverse mutation assay in *Escherichia coli* WP2uvrA at concentrations up to 5000 μg/plate, with and without metabolic activation (Shibuya,

2006).

Negative results were also reported in chromosomal aberrations (CA) and polyploidy assays in Chinese hamster lung cells up to 840 μg/mL (Tanaka, 2006; Kusakabe et al., 2002).

6.6.3.1.2. Substituted thiophenes. No evidence of a mutagenic response was previously reported in *Salmonella typhimurium* with substituted thiophenes, including 2-methyl-, 3-methyl-, 2-acetyl-, and 2,5-dimethyl-thiophenes, 5-methyl-2-thiophenecarboxaldehyde, and 3-thiophene-carboxaldehyde, at concentrations up to 100 μmol/plate with and without metabolic activation (Lee et al., 1994; Aeschbacher et al., 1989). A summary of these studies is shown in Table 7.

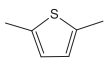
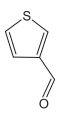
6.6.3.2. Thiophenes positive for mutagenicity. The number and type of thiophene compounds that have shown mutagenic potential are limited. Specifically, only polycyclic thiophenes (3 rings or more) have been positive in Ames mutagenicity tests (Appendix A, Table A-1). Of four 3-ring polycyclic aromatic sulfur heterocycles, only naphtho[1,2-*b*]thiophene was mutagenic in the *Salmonella typhimurium* TA100 strain and much less in the TA98 strain in an Ames assay (Pelroy et al., 1983), whereas the naphtho[2,1-*b*]thiophene, differing only in the position of the sulfur atom, was not mutagenic (Pelroy et al., 1983). Of thirteen 4-ring compounds, seven were mutagenic and phenanthro[3,4-*b*]thiophene had the highest activity, a compound of approximately the same mutagenic potency in the Ames assay as benzo[α]pyrene (Pelroy et al., 1983). A number of polycyclic aromatic sulfur heterocycles, including dibenzothiophene, benzo[β]naphtho[1,2-*d*]thiophene, benzo[β]naphtho[2,1-δ]thiophene and benzo[β]naphtho[2,3-δ]thiophene have demonstrated either weak or no mutagenic activity using the *Salmonella typhimurium* mutagenicity test (Pelroy et al., 1983; McFall et al., 1984).

6.7. Conclusions for toxicity

Substitute thiophene flavoring substances and structurally related compounds have very low acute toxicity. In short term studies with 5-methyl-2-thiophene carboxaldehyde, 3-acetyl-2,5-dimethylthiophene, 5-ethyl-2-thiophene carboxaldehyde and unsubstituted thiophene, no toxicity was observed at intake levels below 100 mg/kg bw/day. In subchronic studies with 2-thienyldisulfide, 2-pentylthiophene and 5-ethyl-2-thiophene carboxaldehyde few adverse effects were reported at high dose levels and the NOAELs derived from these studies provide large margins of safety when compared to estimated levels of exposure from use as flavoring substances (Table 4). The NOAELs for 2-pentylthiophene, 2-thienyldisulfide and 5-ethyl-2-thiophene carboxaldehyde, are applicable to other alkyl-substituted, thiophenes with sulfur in the side chain and acyl-substituted thiophene flavoring substances, respectively. In addition, the intake levels for these thiophene substances are below the toxicological threshold of concern (TTC) for their structural class. Therefore, there are adequate data to support their safety under their specific intended uses as flavor ingredients added to food.

No evidence of genotoxicity has been found for simple substituted thiophenes that are structural analogues of flavoring ingredients (Table 7). Alkyl-substituted thiophene flavoring substances 2-pentylthiophene, 2-hexylthiophene, 3,4-dimethylthiophene as well as 2-thienylmethanol are structurally similar to methylthiophenes (2-, 3-, and 2,5-dimethyl-); the thiophene flavoring substances with sulfur-containing side chains, 2-thienyl mercaptan, 2-thienyldisulfide, 3-(methylthio)-methylthiophene and 1-(2-thienyl)ethanethiol differ from simple alkyl thiophenes due to the presence of the sulfur in the side chain. For the purpose of genotoxicity assessment they are considered structurally similar to simple alkylated thiophenes. 2-Acyl-substituted thiophene flavoring substances 1-(3-hydroxy-5-methyl-2-thienyl)ethanone and 2-acetyl-5-methylthiophene are structurally similar to 2-acetylthiophene and 5-methyl-2-thiophenecarboxaldehyde. Methylthiophenes, 2-acetylthiophene and 3-thiophene-carboxaldehyde (Table 7) and 5-methyl-2-thiophenecarboxaldehyde (Table 6) are negative for genotoxicity. The weak induction of micronuclei that was reported *in vitro* for 5-methyl-2-thiophenecarboxaldehyde was not confirmed when tested *in vivo*. 3-Acyl substituted thiophene 3-acetyl-2,5-dimethylthiophene is structurally similar to 3-thiophene-

Table 7
Summary of *in vitro* and *in vivo* genotoxicity of structurally related thiophene compounds.

Compound	Structure	Assay	Result	Reference
Thiophene		Ames mutagenicity *S. typhimurium* TA98, TA100, TA102, TA1535, TA1537, TA97	Negative	Zeiger et al., 1987a,b Shibuya 2006 Aeschbacher et al., 1989 Lee et al., 1994
		Escherichia coli WP2 *uvr*A reverse mutation	Negative	Shibuya 2006
		Chromosomal aberrations	Negative	Kusakabe et al., 2002 Tanaka 2006
2-Methyl-		Ames mutagenicity *S. typhimurium* TA98, TA100, TA102	Negative	Aeschbacher et al., 1989 Lee et al., 1994
3-Methyl-		Ames mutagenicity *S. typhimurium* TA98, TA100, TA102	Negative	Aeschbacher et al., 1989 Lee et al., 1994
2-Acetyl-		Ames mutagenicity *S. typhimurium* TA98, TA100	Negative	Lee et al., 1994
2,5-Dimethyl-		Ames mutagenicity *S. typhimurium* TA98, TA100	Negative	Lee et al., 1994
3-Thiophene-carboxaldehyde		Ames mutagenicity *S. typhimurium* TA98, TA100	Negative	Lee et al., 1994

carboxaldehyde (Table 4). Whereas, 3-thiophene-carboxaldehyde is negative for genotoxicity, 3-acetyl-2,5-dimethylthiophene tested positive for genotoxicity: it induced mutations in an Ames test in three strains of *Salmonella typhimurium* in the presence of S-9 activation and these *in vitro* results were also confirmed in the Muta™Mouse assay *in vivo*. However, 3-acetyl-2,5-dimethylthiophene was negative for mutagenicity in the absence of metabolic activation *in vitro* and in the *in vivo* micronucleus assay in bone marrow of male rats when dosed up to the MTD. Overall, the data indicate that its genotoxicity is related to an unknown active metabolite generated in the liver. There is no concern about the genotoxic potential of the thiophene flavoring substances evaluated in this report, except for 3-acetyl-2,5-dimethylthiophene. The only other thiophenes that have been reported positive for genotoxicity are polycyclic thiophene derivatives, compounds with very different chemical reactivities than the thiophenes used as flavoring ingredients (Table A-1).

7. Discussion

Thiophene flavoring substances are used at very low levels in food, resulting in low estimated daily intakes. In addition, several thiophene compounds occur naturally in foods. The intake of thiophene substances from natural sources far exceeds the intake from their use as added flavors. Toxicity studies conducted with alkyl, thienyl-, and acyl-substituted thiophenes are representative of the group of thiophene flavoring substances. Together they provide large margins of safety relative to the low estimated intakes from use as flavors added to food.

Genotoxicity data on several substituted thiophenes also support lack of genotoxic potential for the group of thiophene flavoring substances. However, concern for genotoxicity cannot be excluded for one member of this group, 3-acetyl-2,5-dimethylthiophene which displayed mutagenic activity *in vitro* (in the presence of metabolic activation) and *in vivo* (Tables 5–7). While 5-methyl-2-thiophenecarboxaldehyde showed some equivocal evidence of genotoxic potential in the micronucleus test *in vitro*, this was not confirmed in a follow-up *in vivo* micronucleus test suggesting lack of biological relevance of the *in vitro* test. The genotoxic activity of 3-acetyl-2,5-dimethylthiophene stands in contrast to the collective evidence for the remaining thiophene substances in the group and is attributed to the formation of reactive metabolites. The positive mutagenic response of 3-acetyl-2,5-dimethylthiophene was limited to conditions including metabolic activation *in vitro* in the absence of GSH (Ames test + S-9) or in the liver of the Muta™-Mouse, the tissue of maximum metabolic activity. Indeed, the duodenum, site of first contact, did not display a significant mutagenic response to orally administered 3-acetyl-2,5-dimethylthiophene. Ultimately, a carcinogenicity study in rodents would be the most direct way to assess the biological significance of the genotoxicity evidence reported for 3-acetyl-2,5-dimethylthiophene.

A closer look at the metabolic pathways involved in the detoxication of thiophene compounds provides possible reasons for the divergent behavior of the compound in question. It is consistent with the effective formation of a reactive metabolite coupled with inefficient GSH conjugation and elimination.

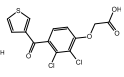
Ring epoxidation and S-oxidation are the known primary pathways for biotransformation of thiophene derivatives, and the relative contribution of each pathway is a function of the thiophene substructure. GSH conjugation of the reactive intermediate seems to be a key element in inhibiting covalent binding by acting as a sink for the reactive metabolites, as demonstrated by Dansette et al., (1990, 2005) and Valadon et al., (1996). When comparing the metabolic fate of thiophene derivatives, a pattern emerges that involves the type (Mansuy et al., 1991), position (Dansette et al., 1990; Valadon et al., 1996; Rademacher et al., 2012) and number of substitution groups. The reactivity increases with a) the presence of a carbonyl group compared to simple aliphatic side chains (Mansuy et al., 1991); b) substitution in position 3 compared to position 2 of the ring (Dansette et al., 1990; Valadon et al., 1996; Rademacher et al., 2012); and c) more extensive substitution (higher number of side groups). The *S*-oxide pathway leads to more reactive intermediates compared to the ring epoxidation pathway. The presence and position of a carbonyl group at C3 renders the *S*-oxide more electrophilic, as indicated by the ratios of stable-to-reactive metabolites of 0.25 and 2, respectively, for 3-aroylthiophene (TAI) and 2-aroylthiophene (TA), following microsomal incubation (Dansette et al., 1990). Furthermore, the S-oxidation is favored in 3-acylthiophenes compared to the 2-acylthiophenes (Rademacher et al., 2012) and position 2 is the most vulnerable to nucleophilic attack (Mansuy et al., 1991; Dansette et al., 1992; Valadon et al., 1996). A comparison of structural features and metabolic outcomes is summarized in Table 8.

Based on these insights from model thiophene structures, it is expected that in the case of 3-acetyl-2,5-dimethylthiophene, the S-oxidation is favored compared to the ring oxidation, due to the position of the acyl group (3- vs 2-), and results in a more reactive intermediate (*S*-oxide intermediates are more reactive compared to ring epoxide intermediates). The metabolic pathway leading to a ring epoxide is less likely to occur in 3-acetyl-2,5-dimethylthiophene, not only because it is 5 times less favored in 3-acyl compared to 2-acyl thiophenes (Dansette et al., 1990) but also because this molecule bears two additional substitution groups compared to the model structure TAI. The dimerization product seen with 2-phenylthiophene would also be less likely for 3-acetyl-2,5-dimethylthiophene for similar reasons. Thus, following CYP activation *in vivo* or *in vitro*, a sulfoxide appears to be the likely reactive metabolite for 3-acetyl-2,5-dimethylthiophene (Fig. 5). In addition, GSH conjugation on the *S*-oxide intermediate of 3-acetyl-2,5-dimethylthiophene, which normally occurs on 2- or 5- positions of 3-substituted model thiophenes, may be less efficient due to the presence of methyl groups on both ring positions 2 and 5. Therefore, detoxication of the reactive intermediate to less reactive and stable products may be less effective for this structure compared to the other substituted thiophenes in the group. However, although GSH binding may be inefficient it should not be presumed to be absent. The *S*-oxide intermediate is an electrophilic molecule and reactive toward nucleophiles, including but not limited to GSH. In the presence of multiple nucleophilic partners, binding can be subject to competition. Unless the electrophile is promptly stabilized by GSH to a less reactive product, the probability of interaction with other macromolecules increases along with the probability of resulting effects.

With regards to 5-methyl-2-thiophenecarboxaldehyde, the presence of the carbonyl group may explain the positive, albeit weak, genotoxic activity *in vitro* compared to absence of genotoxicity of unsubstituted thiophene, or mono- and bi-substituted alkyl thiophenes. The location of the carbonyl group in 5-methyl-2-thiophenecarboxaldehyde on position 2 instead of position 3 may explain the rather weak genotoxic activity observed, presumably due to lower reactivity (low yield of *S*-oxide intermediates) as suggested by the difference in reactivity between the structural analogues TA and TAI (Dansette et al., 1990). Furthermore, conjugation of reactive metabolites with GSH and conversion of reactive metabolites to a carboxylic acid may explain the absence of genotoxicity of 5-methyl-2-thiophenecarboxaldehyde *in vivo*. (Table 8).

Interestingly, two other thiophene flavoring ingredients, 2-acetyl-5-methylthiophene and 1-(3-hydroxy-5-methyl-2-thienyl) ethanone, share similar features with 3-acetyl-2,5-dimethylthiophene, such as the presence of the acyl group and

Table 8
Structural features and metabolic fate of model thiophenes and selected thiophene flavoring substances.

	Reported metabolic fate of model thiophene compounds						Hypothesized metabolic fate of thiophene flavoring ingredients[1]		
Name	**Thiophene**	**TA**	**TAI**	**2-PT**	**BT**	**2-AT**	**3-acetyl-2,5-dimethyl thiophene**	**5-methyl-2-thiophene-carboxaldehyde (FEMA No 3209)**	**1-(3-hydroxy-5-methyl-2-thienyl) ethanone (FEMA No 4142)**
a - Structure									
									
Number of substitution groups	0	1	1	1	fused ring (2,3)	1	3	2	3
Acyl group present	NA	YES	YES	NO	NO	YES	YES	YES	YES
Acyl group position	NA	2	3	NA	NA	2	3	2	2
Position of other substitution groups	NA	NA	NA	2	NA	NA	2, 5	5	3, 5
Ring position available for GSH binding	2, 3, 4, 5	3, 4	2, 5	3, 4, 5	4, 5	3, 4, 5	NO	3, 4	NO
b - Metabolism									
Primary metabolic pathway	Only *S*-Oxide reported [(a)]	Ring epoxide intermediate [(b)]	*S*-Oxide intermediate [(b, c, d, e)]	Ring epoxide intermediate [(f)]	*S*-Oxide intermediate [(d)]	*S*-Oxide intermediate [(c)]	*S*-Oxide intermediate	Ring epoxide intermediate	Ring epoxide intermediate
Secondary metabolic pathway	Ring epoxide likely (NR)	*S*-Oxide implied [(b, f)] but not detected [(c)]	Ring epoxide [(c)]	*S*-Oxide [(f)]	NR	NR	Ring epoxide less likely than for TAI: additional steric hindrance on pos. 2 and 5	*S*-Oxide possible	*S*-Oxide possible
Metabolites detected	2-mercapturate of 2,5-dihydro thiophene-*S*-oxide (30% of dose) [(a)]	5-OH metabolite (65%) [(b, c)]; thiolactone [(c)]	2-thioethanol conjugate of the *S*-oxide [(d)]; 5-OH metabolite (18%) [(b)]; thiolactone isomers; *S*-oxide dimer [(c)]	S-oxide Dimer (2PTSOD); GSH *S*-oxide (2PTGA); GSH OH-product (2PTGB); thiolactone (2PTT) [(f)]	3-thioethanol conjugate of benzothiophene sulfoxide [(d)]	NR	NA	NA	NA
Reactivity of intermediates	NR	Lower (30%) relative to TAI [(b)]	Higher (80%) relative to BT [(d)] and TA [(b)]	Lower in the presence of GSH [(f)]	Lower relative to TAI [(d)]	NR	Similar to TAI	Similar to TA	Similar to TA or lower due to ring OH
Dimer formation	NR	NO [(c)]	YES [(c)]	YES [(f)]	NR	NR	Likely hindered	NO (similar to TA) [(c)]	NO (similar to TA) [(c)]
Ring hydroxylation	NR	YES (65%) [(b, c)]	YES (18%) [(b)]	YES [(f)]	NO [(c)]	NO [(c)]	Only *via* ring epoxide [(c)]	Only *via* ring epoxide [(c)]	Only *via* ring epoxide [(c)]
GSH conjugation	YES (pos. 2)	YES (pos. 4) [(c)]	YES (pos. 2) [(e)]	YES (pos. 4 or 5) [(f)]	YES (pos. 4) [(d)]	NR	Likely hindered	Likely on pos. 4	Expected on existing 3-OH
Experimental data	YES; also *in vivo* [(a)]	YES [(b)]	YES [(b, d)]; and *in vivo* [(e)]	YES [(f)]	YES [(c, d)]	*In silico* [(c)]	NO	NO	NO

[1] Thiophene flavoring substances include 3-acetyl-2,5-dimethylthiophene and two others selected for the presence of similar structural features to highlight the atypical nature of the first relative to the others.
NA: not applicable; NR: not reported;
Blue highlights indicate structural features of 3-acetyl-2,5-dimethylthiophene related to metabolic divergence from other thiophenes
(a) - Dansette and others 1992
(b) - Dansette and others 1990
(c) - Rademacher and others 2012
(d) - Mansuy and others 1991
(e) - Valadon and others 1996
(f) - Dansette and others 2005

substitution on positions 2 and/or 5 of the ring (Table 2). However, both of these compounds are less likely to be as reactive and more likely to be efficiently conjugated for the following reasons: a) both structures have the acyl substituent on position 2 of the ring (compared to position 3), which favors ring oxidation rather than S-oxidation reactions; b) the first structure has only one other substituent, which presents less hindrance for GSH conjugation following ring oxidation; and c) the second structure has a hydroxy substituent on position 3 which is readily available for conjugation reactions.

The relative structural features and the expected metabolic fate of thiophene flavoring ingredients are presented in Table 8.

While the mechanism leading to the genotoxicity of 3-acetyl-2,5-dimethylthiophene is not currently known, the available evidence provides reasonable support for its divergent behavior relative to the other substituted thiophenes in this group of flavoring substances (Table 8). Among all the substituted thiophenes studied to date, most are negative for genotoxicity (Table 7). The only other compounds with genotoxic potential are the polycyclic thiophenes with extended fused ring structures, such as phenanthrol[3,4-*b*]thiophene (Table A-1) (Pelroy et al., 1983). The genotoxicity of those structures are likely related to the other ring substructure rather than to a reactive thiophene intermediate. The flavoring substances reviewed in this report do not possess fused

ring structures and are not considered by the Panel as structurally related to the polycyclic substituted class of thiophenes.

Taken together, the available data on thiophene metabolism and the pattern of mutagenic responses among thiophene compounds indicate that 3-acetyl-2,5-dimethylthiophene presents an atypical case among the group of thiophene flavoring substances, where metabolic transformation favors the formation of a reactive product but not its detoxication at high exposure doses and/or when GSH stores are depleted. Based on the available evidence from a battery of published genotoxicity studies on the thiophene flavor group, this behavior does not present concern for genotoxic potential for the remaining substances in this group.

8. Conclusion

The group of thiophene-based flavoring substances discussed here was previously determined to be generally recognized as safe (GRAS) under conditions of intended use as flavor ingredients by the FEMA Expert Panel (Hall and Oser, 1965, 1970; Oser and Hall, 1972; Oser and Ford, 1978; Smith et al., 2005b, 2009; Waddell et al., 2007). In 1978, the Panel evaluated the available data and affirmed the GRAS status of all previously GRAS flavor ingredients (GRASa). In 1993, the Panel initiated a comprehensive program to re-evaluate the status of all FEMA GRAS flavor ingredients concurrent with a systematic revision of the FEMA Scientific Literature Reviews (available from the National Technical Information Service, NTIS) and the Panel reaffirmed the status of 4 members of this group in 2001 with GRASr status. In the interim, new data have become available for two substances, 5-methyl-2-thiophenecarboxaldehyde and 3-acetyl-2,5-dimethylthiophene, in recent genotoxicity studies conducted according to OECD guidelines. The new studies did not present any concern for genotoxicity for 5-methyl-2-thiophenecarboxaldehyde. In contrast, results of *in vitro* and *in vivo* studies for 3-acetyl-2,5-dimethylthiophene raise a concern for genotoxicity. The genotoxicity of 3-acetyl-2,5-dimethylthiophene in the assays is unexplained mechanistically, however, based upon knowledge of the metabolic fate of thiophenes, it appears to be related to specific metabolic differences as a result of structural features unique to this substance compared to the remaining thiophene substances in the group.

The FEMA GRAS status of 3-acetyl-2,5-dimethylthiophene (formerly FEMA No 3527) under conditions of intended use as a flavor ingredient was reviewed by the Panel. Based on the available scientific evidence related to safety, the Panel concluded that additional data, including more detailed exposure information, comparative metabolism studies, and more comprehensive toxicity data including an in-depth evaluation of the mechanism of action for potential adverse effects are required. Until such data are available for review, the flavor ingredient 3-acetyl-2,5-dimethylthiophene has been removed from the GRAS list. The Panel also reviewed data for related alkyl-substituted thiophene substances and confirmed that no change in the GRAS status of their uses was warranted.

Overall there is sufficient available genetic toxicity testing data to indicate that, with the exception of the flavoring substance 3-acetyl-2,5-dimethylthiophene, the group of the 11 remaining thiophene-based flavoring substances does not present any concern with respect to genotoxicity. Therefore, the Expert Panel finds that the remaining 11 thiophene flavoring substances are reaffirmed as GRAS based on their rapid absorption, metabolic conversion, and excretion in humans and animals; their low levels of use as flavors in food; the wide margins of safety between the conservative estimates of intake and the experimentally determined NOAEL or NOELs derived from repeat dose subchronic oral toxicity studies in rodents as well as a lack of significant, biologically relevant genotoxic potential. The consistency of the results obtained from appropriately conducted *in vivo* subchronic toxicity studies in rodent models at high doses support the conclusion that consumption of these thiophene substances under intended use conditions as flavors in food is not a concern for human health.

Author contributions

Drs. Cohen, Fukushima, Gooderham, Guengerich, Hecht, Rietjens, and Smith interpreted the results and drafted the manuscript. Ms. Harman, Drs. Bastaki, McGowen, Valerio, and Taylor researched and compiled available study data and assisted in the drafting of the manuscript.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.10.023.

Appendix A

Table A-1
Summary of *in vitro* genotoxicity of polycyclic thiophene compounds.

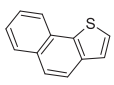
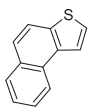
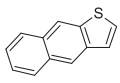
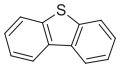
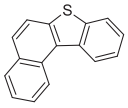
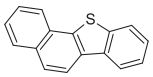
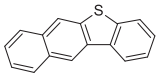
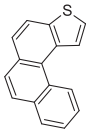
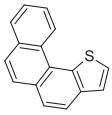
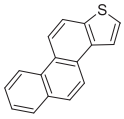
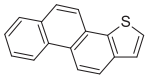
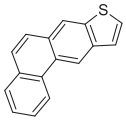
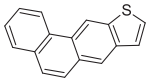
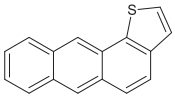
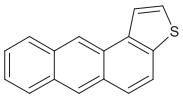
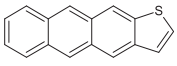
Compound	Structure	Assay	Result	Reference
Naphthol[1,2-*b*]thiophene		Ames mutagenicity	Positive [a, b, c, d]	Pelroy et al., 1983
Naphthol[2,1-*b*]thiophene		Ames mutagenicity	Negative	Pelroy et al., 1983
Naphthol[2,3-*b*]thiophene		Ames mutagenicity	Negative Weakly pos.[a, c, d] Weakly pos.[e, f, d]	Pelroy et al., 1983

Table A-1 (*continued*)

Compound	Structure	Assay	Result	Reference
Dibenzothiophene		Ames mutagenicity	Negative Weakly pos.[a, g, d] Weakly pos.[b, f, c]	Pelroy et al., 1983 McFall et al., 1984
Benzo[*b*]naphtho[1,2-*d*]thiophene		Ames mutagenicity	Negative	Pelroy et al., 1983 McFall et al., 1984
Benzo[*b*]naphtho[2,1-*d*]thiophene		Ames mutagenicity	Weakly pos.[a, b, d] Weakly pos.[e, f, d] Weakly pos.[b, f, c]	Pelroy et al., 1983 McFall et al., 1984
Benzo[*b*]naphtho[2,3-*d*]thiophene		Ames mutagenicity	Negative	Pelroy et al., 1983 McFall et al., 1984
Phenanthrol[3,4-*b*]thiophene		Ames mutagenicity	Positive [a, b, c, d]	Pelroy et al., 1983
Phenanthrol[4,3-*b*]thiophene		Ames mutagenicity	Negative	Pelroy et al., 1983
Phenanthrol[2,1-*b*]thiophene		Ames mutagenicity	Weakly pos.[d, e, a, f]	Pelroy et al., 1983
Phenanthrol[1,2-*b*]thiophene		Ames mutagenicity	Weakly pos.[e, f, d]	Pelroy et al., 1983
Phenanthrol[2,3-*b*]thiophene		Ames mutagenicity	Weakly pos.[e, f, d]	Pelroy et al., 1983
Phenanthrol[3,2-*b*]thiophene		Ames mutagenicity	Weakly pos.[e, f, d] Weakly pos.[b, f, c]	Pelroy et al., 1983
Anthra[1,2-*b*]thiophene		Ames mutagenicity	Positive [a, b, c, d] Positive [b, f, c] Positive [e, f, d]	Pelroy et al., 1983
Anthra[2,1-*b*]thiophene		Ames mutagenicity	Positive [a, b, c, d] Positive [b, f, c] Positive [e, f, d]	Pelroy et al., 1983
Anthra[2,3-*b*]thiophene		Ames mutagenicity	Positive [a, b, c, d]	Pelroy et al., 1983

[a] Plate incorporation method.
[b] With S-9.
[c] Strain TA98.
[d] Strain TA100.
[e] Without S-9.
[f] Pre-incubation method.
[g] With and without S-9.

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