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Review

Analytical methods for trace levels of reactive carbonyl compounds formed in lipid peroxidation systems

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Abstract

Analysis of trace levels of reactive carbonyl compounds (RCCs), including formaldehyde, acetaldehyde, acrolein, malonaldehyde, glyoxal, and methyl glyoxal, is extremely difficult because they are highly reactive, water soluble, and volatile. Determination of these RCCs in trace levels is important because they are major products of lipid peroxidation, which is strongly associated with various diseases such as cancer, Alzheimer's disease, aging, and atherosclerosis. This review covers the development and application of various derivatives for RCC analysis. Among the many derivatives which have been prepared, cysteamine derivatives for formaldehyde and acetaldehyde; *N*-hydrazine derivatives for acrolein, 4-hydroxy-2-nonenal, and malonaldeyde; and *o*-phenylene diamine derivatives for glyoxal and methyl glyoxal were selected for extended discussion. The application of advanced instruments, including gas chromatograph with nitrogen–phosphorus detector (GC/NPD), mass spectrometer (MS), high performance liquid chromatograph (HPLC), GC/MS, and LC/MS, to the determination of trace RCCs in various oxidized lipid samples, including fatty acids, skin lipids, beef fats, blood plasma, whole blood, and liver homogenates, is also discussed.
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Keywords: Acetaldehyde; Acrolein; Formaldehyde; Gas chromatography; Glyoxal; Lipid peroxidation; Methyl glyoxal; Reactive carbonyl compounds; Trace analysis

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1. Introduction

Trace or ultra-trace analysis of low molecular weight carbonyl compounds – so called reactive carbonyl compounds (RCCs) – such as formaldehyde, acetaldehyde, acrolein,

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malonaldehyde (MA), glyoxal, and methyl glyoxal, is one of the most difficult experimental procedures. These RCCs are significantly reactive and form adducts readily with biological substances such as proteins, phospholipids, and DNA. It is extremely difficult to recover them from a biological matrix because they are highly water soluble.

It is, however, very important to analyze these RCCs in trace levels because they are major products of lipid peroxidation and consequently present in various biological substances. Moreover, lipid peroxidation is strongly associated with various diseases, including cancer [1–4], mutagenesis [5,6], Alzheimer's [7–13], aging [14,15], arthritis [16,17], inflammation [18,19], diabetes [20–22], atherosclerosis [23–25], and AIDS [26–31].

Some RCCs formed from lipids by oxidative damage have been used as biomarkers in order to investigate their roles in the diseases mentioned above. In order to obtain satisfactory results from these investigations, however, accurate and reliable and, ideally, simple methods for RCC analysis are required. In this review, the development and application of various derivatives for trace analysis of RCCs are summarized. The use of advanced instruments, including gas chromatograph with nitrogen–phosphorus detector (GC/NPD), mass spectrometer (MS), high performance liquid chromatograph (HPLC), GC/MS, and LC/MS, for the determination of trace levels of RCCs in various oxidized lipid samples, such as fatty acids, skin lipids, beef fats, blood plasma, whole blood, and liver homogenates, is also discussed.

2. Formation of lipid peroxidation products

The oxidative degradation of lipids has been studied from various perspectives such as its roles in the alteration of foods, thermal oxidation, autoxidation, and oxidation with reactive oxygen species. In order to initiate lipid peroxidation, a lipid molecule must be activated by an initiator. The most common initiators are reactive oxygen species (ROSs). They are superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), triplet oxygen ($^3O_2$), hydroxy radical ($^{\bullet}OH$), alkoxy radical ($RO^{\bullet}$), and peroxy radical ($ROO^{\bullet}$). These ROSs play an important role in lipid peroxidation. There have been many reports on proposed mechanisms of lipid peroxidation, which produces many RCCs. One of the most well known mechanisms is the autoxidation of unsaturated fatty acids such as linoleic acid, linolenic acid, arachidonic acid, and various ω-3 fatty acids [32–41].

The basic mechanisms of autoxidation can be found in many reference books on food science [42–46]. The autoxidation of unsaturated fatty acids occurs slowly, initiated by a triplet oxygen ($^3O_2$) [47]. An ROS abstracts a hydrogen atom from a methylene group of an unsaturated fatty acid and subsequently forms free radicals such as a peroxyl radical [48]. Once these free radicals are formed, lipid peroxidation progresses and, consequently, many secondary oxidation products are formed.

A singlet oxygen ($^1O_2$) is highly electrophilic and reacts readily with unsaturated fatty acids. However, its mechanism is different from that of free radical autoxidation. The most common oxidation of lipids involving singlet oxygen is photo-oxidation [49]. In order to undergo photochemical reaction in biological lipids, certain photosensitizers such as chlorophyll, porphyrins, myoglobin, and riboflavin are required. Therefore, natural substances containing these photosensitizers tend to undergo lipid peroxidation upon UV irradiation. In particular, unsaturated lipids in cell membranes, including phospholipids and cholesterol, are well-known targets of oxidative modification, which can be induced by a variety of stresses, including UVA and visible light-induced photodynamic stress [50]. There are many reports on the formation of RCCs from UV irradiated lipids such

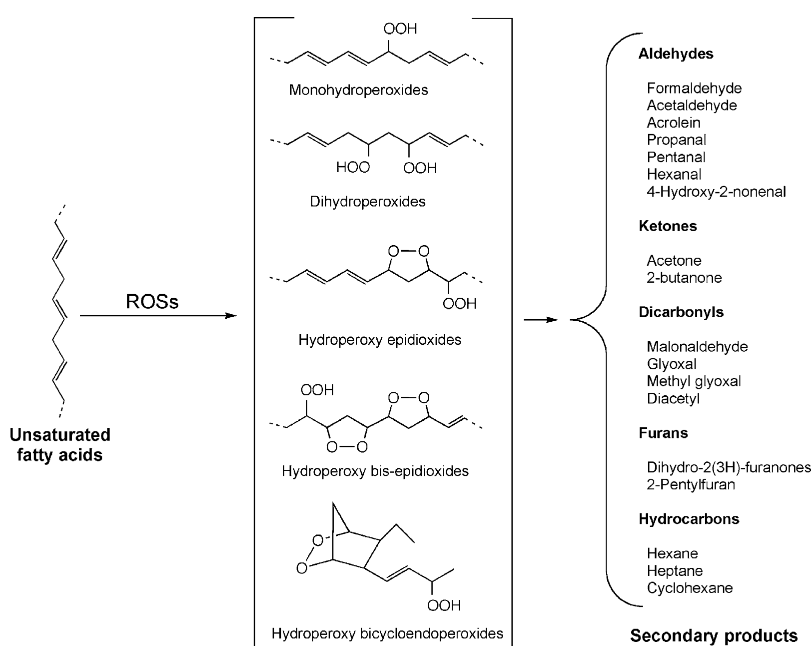

Fig. 1. Proposed intermediates and secondary products formed from lipids by oxidation.

Table 1
Reactive carbonyl compounds (RCCs) discussed in this review

RCC	Formula	B.P.	M.W.	Water solubility (%)	Derivatized by	Derivative
Formaldehyde	HCHO	−19.5	30.03	55	Cysteamine	Thiazolidine
Acetaldehyde	CH_3CHO	21	44.05	100	Cysteamine	2-Methylthiazolidine
Acrolein	$CH_2{=}CHCHO$	52.5	56.0	67	*N*-Methylhydrazine	1-Methyl-2-pyrazoline
4-Hydroxy-2-nonenal	$CH_3(CH_2)_4CH(OH)$-$CH_2{=}CH_2CHO$	275.6	156.22	Slightly	*N*-Methylhydrazine	5(1′-Hydroxyhexyl)-1-methyl-2-pyrazoline
Malonaldehyde	$OHCCH_2CHO$	108.3	72.06	100	*N*-Methylhydrazine	1-Methylpyrazole
Glyoxal	OHCCHO	50.4	58.04	Soluble	*o*-Phenylene diamine	Quinoxaline
Methyl glyoxal	CH_3COCHO	72.0	72.06	Soluble	*o*-Phenylene diamine	2-Methylquinoxaline

as corn oil [51], squalene [52,53], linoleic and linolenic acids [54], cod liver oil [55], ethyl arachidonate [56], and triolein [57].

Heat treatments, including cooking and processes for food product preparation, cause oxidation of food components and, in particular, of lipids. The oxidation of lipids by heat may involve different mechanisms from those of autoxidation and photo-oxidation because the conditions of thermal oxidation are much more intense than those of autoxidation or photo-oxidation [58]. Therefore, many secondary compounds have been identified from thermally treated lipids, including beef fat [59,60], cooking oils [61,62], pork fat [63,64], and dietary oils [65].

Fig. 1 shows the formation mechanisms of secondary products formed from lipids via oxidation that have been reported previously [66,67]. Table 1 shows typical RCCs formed from lipid peroxidation and their physical constants. Some secondary lipid peroxidation products, such as MA, have been widely used as biomarkers for investigating in vivo and in vitro oxidative damage [54–59,61,63,64,68–70]. Even though numerous lipid peroxidation products have been identified, only a few chemicals have been satisfactorily used as biomarkers of oxidative damages.

3. Toxicity of RCCs

It is well-known that oxidative damage, in particular, lipid peroxidation, is strongly associated with various diseases, as mentioned above [71]. There have been many reports on the toxicity of oxidized lipids and the formation of toxic compounds from oxidized lipids [72–74]. For example, oxidized methyl linoleate, containing 4-hydroxy-2-nonenal (4-HN) as the major component, caused lymphocyte necrosis in the thymus and Peyer's patches in mice [75]. Palm oil oxidized by heat caused reduced rates of pregnancy (by 55%) in rats [76].

The toxicity of oxidized lipids is caused by the interaction of secondary products of RCCs rather than ROSs directly, because ROSs are not readily absorbed by the intestines [77]. Among the many products of lipid peroxidation, RCCs such as formaldehyde, acetaldehyde, acrolein, MA, glyoxal, and methyl glyoxal have received much attention as the chemicals implicated in various diseases [78,79]. The oral toxicity of formaldehyde has been reviewed extensively [80]. Formaldehyde has shown potential carcinogenicity in animal studies [81]. Alterations in biological proteins were observed in the lungs of rats after they were exposed to gaseous formaldehyde at 32–37 mg/m^3 for 4 h/day for 15 days [82].

Irritancy is a property of nearly all the aldehydes, in particular of low molecular weight aldehydes. However, acetaldehyde is much less irritating to the human eye, nose, and throat than formaldehyde or acrolein. The chronic toxicities of acetaldehyde, such as carcinogenicity, have not been defined by appropriate long-term animal studies. Spindle-cell sarcomas were produced in rats given acetaldehyde by subcutaneous injections but metastasis to other tissues was not observed [83]. An inhalation toxicity study on acetaldehyde resulted in 23 of the 59 mice dying by exposure to 10 mg/L for 2 h [84]. Studies using cultured human cells indicate that mM concentration levels of acetaldehyde cause a wide range of cytopathic effects associated with multistep carcinogenesis [85].

It is well-known that oxidized lipids yield acrolein and, in fact, that high levels of it are produced because it forms from glycerol after the hydrolysis of triglycerides upon oxidation [61]. Acrolein is also known as a strong eye irritant. A comprehensive review on acrolein – its environmental occurrence and fate, its chemistry, and its toxicology – has been published [86]. There have also been reports on acrolein's mutagenicity in the last two decades [87]. Investigation of oxidative stress associated with lipid peroxidation indicated that acrolein was approximately 100 times more reactive than 4-HN and is present in neurfibrillary tangles in the brain of Alzheimer's disease patients [88].

MA may be the best known lipid peroxidation product and the one that has been used most widely as a biomarker for various studies associated with lipid peroxidation. However, its toxicity has not yet been well established. The fact that MA reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine, subsequently implicates it in mugagenicity and carcinogenicity [89]. The main MA/DNA adducts were reportedly M_1G {pirymido [1,2a]puri-10(3H)-one} and M_1A {N^6-(3-oxo-propenyl)deoxyadenosine}. The amount of M_1G was approximately five times greater than that of M_1A [90–92]. When 500 μg MA/g body weight was administered to 8-week-old female Swiss mice, pancreatic lesions consisting primarily of atrophied exocrine cells with loss of zymogen granulation occurred [93].

A study using male outbreed Wistar rats indicated that glyoxal exerted tumor-promoting activity on rat glandular stomach carcinogenesis [94]. Methyl glyoxal is also reported to have

various biological implications [95]. Development of stomach neoplasms was observed in 6% of experimental animals (mice), which were administered 10 μg methyl glyoxal/g body weight [96]. Methyl glyoxal inhibited protein, DNA, and RNA synthesis in villus and crypt cells as well as colonocytes [97]. These reports clearly indicate that some dicarbonyl compounds produced from lipid peroxidation caused genotoxicities in experimental animals.

There have been numerous studies on toxicities of RCCs in addition to the reports described above; review of these RCC toxicities falls outside the scope of this review.

4. Analytical methods for RCCs formed from lipid peroxidation

As mentioned above, several RCCs have been used as biomarkers for investigations of the relationships between lipid peroxidation or oxidative damage and various diseases. Therefore, numerous studies have been conducted to develop and improve the analytical methods for lipid peroxidation products. Table 2 shows the limits of detection (GC/NPD), MS and NMR spectra data relative to RCC derivatives. The RCCs listed in the table are the ones that will be discussed in this review.

4.1. Application of 2,4-dinitrophenyl hydrazine (DNPH) derivatives

Due to the lack of appropriate analytical methods or sample preparation methods, the presence of trace levels of formaldehyde in lipid peroxidation products was not reported until the 1990s. Low-molecular-weight aldehydes, such as formaldehyde, acetaldehyde, acrolein, MA, are extremely difficult to extract from an aqueous solution with an organic solvent because they are soluble both in water and in lipids. It is, therefore, necessary to prepare more stable and less reactive derivatives. The most widely used derivatives for analysis of RCCs are DNPH derivatives (Fig. 2). Over 90% of the studies associated with RCCs produced from lipid peroxidation have involved the use of DNPH derivatives. There have, therefore, been numerous review articles on the use of DNPH derivatives for analysis of RCCs in various samples, including air, water, foods, tissues, and blood [102–106]. Once RCCs are derivatized into their corresponding hydrazones, they are analyzed by various methods, including spectrophotometry, gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), HPLC, and HPCL/MS. For example, MA, formaldehyde, acetaldehyde, and acetone were detected in rat heart reperfusates by HPLC after they were derivatized with DNPH to the corresponding hydrazone (RCC-DNPH adduct), followed by *n*-pentane extraction [107]. The amounts of RCCs recovered in this study ranged from 0.08 ± 0.02 to 0.13 ± 0.02 nmol/mL for MA-DNPH; from 2.98 ± 0.97 to 4.21 ± 0.63 nmol/mL for formaldehyde-DNPH; from 3.69 ± 1.03 to 6.15 ± 1.12 nmol/mL for acetaldehyde-

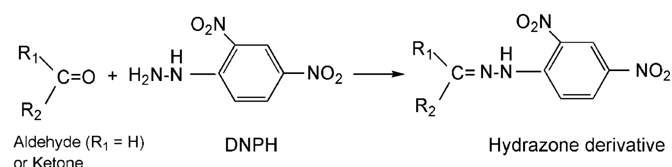

Fig. 2. Formation of hydrazone derivatives from aldehydes or ketones with 2,4-dinitrophenyl hydrazine (DNPH).

Table 2
Limits of detection (GC/NPD), MS and NMR spectra data relative to RCC derivatives

Derivative	LOD[a] (pg)	MS, *m/z* (%)	^{1}H NMR, δ (ppm)	Refs.
Thiazolidine	17.2	M^+ = 89 (95), 59 (23), 43 (100).	δ 1.84 (1H, s, N*H*), 2.82 (2H, t, S-CH_2-CH$_2$), 3.17 (2H, t, CH$_2$-CH_2-N), 4.12 (2H, s, S-CH_2-N)	[98]
2-Methylthiazolidine	16.2	M^+ = 103 (80), 88 (95), 56 (100), 43 (75)	δ 1.52 (3H, d, CH$_2$-CH_3), 1.68 (1H, s, N*H*), 2.90–3.50 (4H, m, S-CH_2-CH_2-N), 4.49 (1H, q, N(S)C*H*-CH$_3$)	[98]
2-Methyl-2-pyrazoline	8.9	M^+ = 84 (79), 83 (100), 56 (38), 43 (31), 42 (67), 41 (17)	δ 2.66 (2H, t, *J* = 9.1 Hz), 2.82 (3H, s), 2.49 (2H, t, *J* = 9.1 Hz), 6.79 (1H, s)	[99,54]
5(1′-Hydroxyhexyl)-1-methyl-2-pyrazoline[b]	8.4	M^+ = 184, 83 (100), 42 (86), 84 (52), 56 (42), 41 (41)	δ 6.75 (1H, brs), 3.84 (1H, brs), 2.93 (1H, ddd, *J* = 2.5, 10.0, 13.0 Hz), 2.83 (3H, s), 2.80 (1H, ddd, *J* = 1.5, 13.0, 16.5 Hz), 2.58 (1H, ddd, *J* = 1.8, 10.0, 16.5 Hz), 2.33 (1H, brs), 1.5 (2H, m), 1.3 (6H, m), 0.90 (3H, t, *J* = 7.5 Hz)	[68]
5(1′-Hydroxyhexyl)-1-methyl-2-pyrazoline[c]	8.4	M^+ = 184, 83 (100), 42 (85), 84 (44), 41 (41), 56 (41)	δ 6.71 (1H, brs), 3.53 (1H, brs), 3.0 (1H, ddd, *J* = 5.0, 10.5 11.0 Hz), 2.90 (1H, ddd, *J* = 2.0, 10.5, 15.5 Hz), 2.87 (3H, s), 2.63 (1H, ddd, *J* = 2.0, 10.5, 15.5 Hz), 2.21 (1H, brd, 1.5 (2H, m), 1.3 (6H, m), 0.90 (3H, s)	[68]
1-Methylpyrazole	7.8	M^+ = 82 (100), 54 (65), 53 (47), 41 (35)	δ 3.80 (3H, s), 6.12 (1H, dd, *J* = 1.8, 2.2 Hz), 7.22 (1H, d, *J* = 2.6 Hz), 7.38 (1H, m)	[51]
Quinoxaline	5.2	M^+ = 130, 103 (61), 76 (49), 50 (21)	δ 7.50 (2H, dd, *J* = 7.5, 3.0 Hz), 7.90 (2H, dd, *J* = 7.5, 3.0 Hz, aromatic H's), 8.61 (1H, s, N = C*H*)	[56,65,100]
2-Methylquinoxaline	13.0	M^+ = 144 (100), 117 (81), 76 (49), 50 (23)	δ 2.38 (3H, s, CH_3), 7.34 (2H, m, aromatic H's), 7.71 (2H, aromatic H's), 8.34 (1H, s, N = C*H*)	[56,65,101]

[a] Limit of detection by GC/NPD.
[b] Diasteromers.
[c] Diasteromers.

DNPH; and from 19.20 ± 1.5 to 28.23 ± 2.12 nmol/mL for acetone-DNPH. RCCs (MA, formaldehyde, acetaldehyde, acetone, and propionaldehyde) in blood samples were also analyzed using RCC-DNPH adducts, followed by HPLC/photodiode array detection [108].

Application of GC to RCC-DNPH adducts analysis is somewhat difficult because most RCC-DNPH adducts are less volatile. However, resolution and sensitivity are still better by GC than by HPLC. The GC/MS analysis of several C_4, C_5, and C_6 isomeric carbonyl compounds formed in cigarette mainstream smoke was performed using their DNPH adducts [109]. After C_1–C_{12} carbonyl compounds in emission samples were trapped on DNPH-coated cartridges, the front traps were analyzed by GC/MS after thermal desorption and then the back-up cartridges were analyzed by HPLC/UV after liquid extraction [110]. Recently, the application of LC/MS to the analysis of RCC-DNPH adducts has become increasingly common due to improvements in LC/MS.

In the mid-1980s, some RCCs were analyzed as RCC-DNPH adducts by a prototype HPLC/MS [111] via a moving-belt interface using a negative ion mode. Detection of nanogram amounts of carbonyl compounds, including formaldehyde, acrolein, propionaldehyde, and acetone, could be achieved by this method [112]. Since the development of a reliable commercial electron spray LC/MS, it has become the mainstream method for the analysis of RCC-DNPH adducts. For example, sub-ppb sensitivity and good reproducibility were obtained for RCC-DNPH adducts, including formaldehyde, acetaldehyde, acrolein, and acetone, analyzed using LC/MS [113]. Disinfected water (from outdoor swimming pools after chlorination) was analyzed for aldehydes, including RCCs, as DNPH-adducts using HPLC/electrospray ionization (ESI)/tandem mass spectrometry (LC/MS/MS) without sample pre-concentration. In this study, detection limits in the μg/L range were achieved by selected ion monitoring measurements (e.g., limits of detection and quantification for acetaldehyde were 0.18 and 0.65 μg/L, respectively)[114].

4.2. Application of pentafluorophenyl hydrazine (PFPH) derivatives

PFPH derivatives (Fig. 3) are more volatile than DNPH derivatives and are more suited to GC analysis [115]. Moreover, because PFPH derivatives contain five fluorine atoms, a highly selective and sensitive electron-capture detector (ECD) or GC/MS with negative chemical ionization MS (NCI/GC/MS) can be used [116]. Trace levels of MA in biological fluids, including human plasma, sperm cells, and cell culture samples, were successfully analyzed using a PFPH derivative, followed by GC/MS. Basal levels of MA found in various normal biological samples were 25–38 pmol/mL human plasma, 0.7–0.8 nmol/g rat liver tissue, and 0.4–3.9 pmol/mg sperm protein [117,118]. Detection limits of femtomole levels for some RCCs were determined using the GC/ECD method [119]. In this study, detection limits of PFPH-acrolein adduct by various detectors were reported: 16.60 fmol/mL by using ECD, 1.31 pmol/mL by using MS-SIM, 1.25 nmol/mL by using nitrogen–phosphorus detector (NPD), and 20.54 nmol/mL by using flame ionization detector (FID). Comparison studies between PFPH/GC and DNPH/GC methods applied to vapor-phase RCCs (formaldehyde, acetaldehyde, acrolein, and butanal) showed that lower detection limits were afforded by the PFPH/GC method [120]. This PFPH/GC method has been used particularly in headspace analysis of RCCs in various foods and beverages, which were trapped on PFPH impregnated cartridges [121,122].

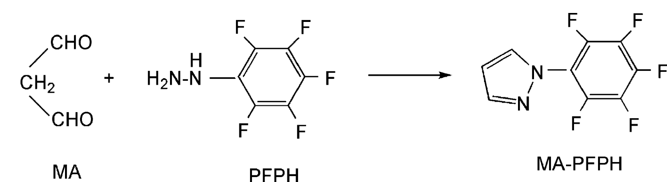

Fig. 3. Formation of MA-penta-fluorophenyl hydrazine (PFPH) adduct from MA.

4.3. Application of newly developed cysteamine derivatives to mono-aldehydes analysis

Recently, several new derivatives have been prepared to aid in the analysis of trace levels of RCCs formed in various samples associated with lipid peroxidation [123–125]. Monoaldehydes such as formaldehyde and acetaldehyde were derivatized to thiazolidine derivatives with cysteamine (Fig. 4) and then analyzed by GC/NPD [126] or GC/FPD [127,128]. This derivative has several advantages over other derivatives: only one derivative is formed from formaldehyde (DNPH produces two isomers); the derivatization reaction occurs rapidly under mild conditions (at room temperature and pH 7) with high and stoichiometric yield; the derivatives of RCCs are stable and reasonably volatile and can be separated perfectly from complex matrices with a GC column; excess of the reagent, cysteamine, does not interfere with GC analysis.

RCCs are formed from lipid components in foods and beverages when they are oxidized [66,67]. Therefore, deterioration of foods and beverages caused by oxidation can be investigated by monitoring formation of an RCC. For example, acetaldehyde in beer samples was analyzed by a cysteamine/GC/NPD

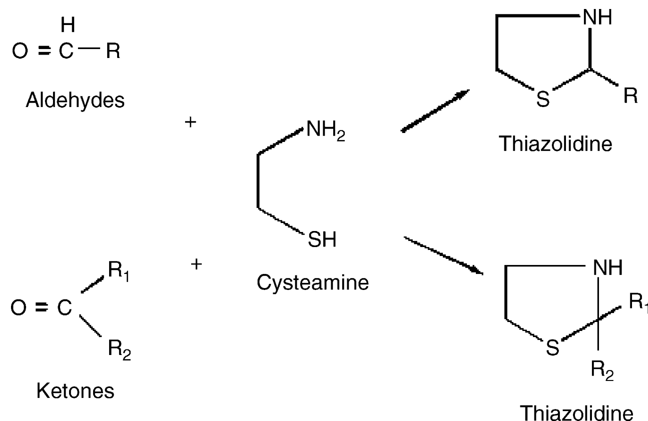

Fig. 4. Formation of thiazolidine derivatives from aldehydes or ketones with cysteamine.

Table 3
Amounts of formaldehyde and acetaldehyde found in various samples analyzed using cysteamine derivatives

Sample	Oxidation method	Amount	Refs.
Formaldehyde			
Brewed coffee (reg.)	Heat	4.5–4.9 ppm	[131]
Brewed coffee (decaf.)		3.4 ppm	[131]
Cocoa	Heat	3.0 ppm	[132]
Instant tea	Heat	3.0 ppm	[132]
Instant coffee	Heat	10–16.3 ppm	[132]
Pork fat	Heat	7.99–25.2 mg/L headspace	[63]
Arachidonic acid	Fenton	115 nmol/mg acid	[69]
Ethyl arachidonate	Fenton	49.4 nmol/mg ester	[69]
Linolenic acid	Fenton	106.6 nmol/mg acid	[69]
Ethyl linolenate	Fenton	141.5 nmol/mg ester	[69]
Linoleic acid	Fenton	59.3 nmol/mg acid	[69]
Ethyl linoleate	Fenton	104.1 nmol/mg ester	[69]
Oleic	Fenton	100.9 nmol/mg aced	[69]
Ethyl oleate	Fenton	119.7 nmol/mg ester	[69]
Fresh corn oil	Heat	53 μg/L headspace	[62]
Used corn oil	Heat	72 μgL headspace	[62]
Cotton seed oil	Heat	99 μg/L headspace	[62]
Sunflower oil	Heat	85 μg/L headspace	[62]
Ambient air	–	48.9–56.2 ppb (v/v)	[133]
Ethyl arachidonate	Microwave	7.0–8.8 nmol/mg ester	[134]
Ethyl linolenate	Microwave	5.8–8.4 nmol/mg ester	[134]
Ethyl linoleate	Microwave	4.4–5.3 nmol/mg ester	[134]
Ethyl arachidonate	Heat	6.1–9.6 nmol/mg ester	[134]
Ethyl linolenate	Heat	8.8–10.4 nmol/mg ester	[134]
Ethyl linolenate	Heat	3.2–4.5 nmol/mg ester	[134]
Squalene	UV	3.4 nmol/mg squalene	[53]
Expired air from mice	–	898 nmol/kg.bw (control)	[135]
Expired air from mice	–	−1356 nmol/kg.bw (tumor bearing)	
Triolein	UV	0.09–6.05 nmol/mg triolein	[57]
Auto-exhaust gases	–	1.32–6.5 μg/L gas	[136]
Fish flesh	Heat	0.48 (mackerel)–5.31 (sardine) μg/g flesh	[137,138]
Cigarette smoke	–	73.8–28.3 μg/cigarette	[139]
Exhaled air from cancer patients	–	0.45–1.2 ppm (cancer/bw)	[140]
MTBE	UV	2.12 μmol/40 mL MTBE	[141]
Zousoon (pork bundle)	Heat	7–62 ppm	[124]
Cigarette smoke	–	87–243 μg/cigarette	[101]
Acetaldehyde			
Arachidonic acid	Fenton	4.02 nmol/mg acid	[69]
Ethyl arachidonate	Fenton	23.0 nmol/mg ester	[69]
Linolenic acid	Fenton	22.7 nmol/mg acid	[69]
Ethyl linolenate	Fenton	20.9 nmol/mg ester	[69]
Linoleic acid	Fenton	67.3 nmol/mg acid	[69]
Ethyl linoleate	Fenton	58.9 nmol/mg ester	[69]
Oleic acid	Fenton	46.4 nmol/mg acid	[69]
Ethyl oleate	Fenton	17.4 nmol/mg ester	[69]
2-Dioxy cytidine	Fenton	125 nmol/16 μmol cytidine	[69]
Thymidine	Fenton	111 nmol/16 μmol thymidine	[69]
2′-Deoxyguanosine	Fenton	90.5 nmol/16 μmol guanosine	[69]
2′-Deoxy adenosine	Fenton	89.9 nmol/16 μmol adenosine	[69]
Human blood	Fenton	6.17 μmol/mL blood	[130]
Aminal bloods	Fenton	5.02 (sheep)–14.8 μmol/mL blood (pig)	[130]
LDL	Fenton	2.25 nmol/10.9 μg LDL	[142]
Blood plasma	Fenton	135 nmol/516 μg plasma	[142]
L-Ascorbic acid	Fenton	694.8 nmol/g acid	[143]
D-Erythrose	Fenton	308.3 nmol/g sugar	[143]
L-Threonic acid	Fenton	204.7 nmol/g acid	[143]
Cigarette smoke	Fenton	1491–2705 μg/cigarette	[139]
Fish flesh	Fenton	1.70 (squid)–15.47 μg/g flesh (sardine)	[137,138]
Auto-exhaust gases	Fenton	1.32–6.60 μg/L gas	[144]
Spleen (mice)	–	6.56 (control)–12.51 nmol/g bw (tumor bearing)	[145]
Expired air (mice)	CCl_4	53.6–54.6 μg (control) to 44.4–55.6 μg (treated)	[146]
Triolein	UV	0.06–2.71 nmol/mg triolein	[57]

Table 3 (*Continued*)

Sample	Oxidation method	Amount	Refs.
Expired air (mice)	–	1494 (control)–859 mg/L air (tumor bearing)	[135]
Squalene	UV	10.37 nmol/mg squalene	[53]
Cod liver oil	UV	3.5–49.1 nmol/mg oil	[55]
Corn oil	Heat	583 (fresh)–859 μg/L headspace (used)	[62]
Cotton seed oil	Heat	576 μg/L headspace	[62]
Sunflower oil	Heat	1130 μg/L headspace	[62]
Soybean/sesame oils	Heat	572 μg/L headspace	[62]
Pork fat	Heat	49.1–339 mg/L headspace	[63]
Zouzoon	Heat	6–69 ppm	[124]
Cigarette smoke	Heat	1110–2101 μg/cigarette	[10]

method to investigate the shelf-life of beer [129]. Trace levels of acetaldehyde present in samples of blood from 10 animals, including human blood, were analyzed using a cysteamine derivative. The quantities of acetaldehyde found in blood ranged from 2.04 μmol/mL (hamster) to 14.8 μmol/mL (pig). The quantity of acetaldehyde recovered from human blood was 6.17 μmol/mL in this study [130]. Table 3 shows a summary of formaldehyde and acetaldehyde analyses reported in various articles.

4.4. Methods for recovery of derivatives from lipid-rich samples

As mentioned above, isolation or sample preparation of RCCs from lipid or lipid-rich samples, including foods, animal blood, tissue, and natural plants, for instrumental analyses is extremely difficult. Recently, solid phase extraction (SPE) is becoming more popular than solvent extraction for recovering RCCs from lipid samples due to drastic improvements in commercial SPE [147]. However, the capacity of an SPE cartridge is rather low and it is readily contaminated with undesirable materials. In particular, lipid materials tend to accumulate on SPE and damage the resolution of cartridges. Therefore, these cartridges are used mainly for relatively clean samples such as air samples from atmospheric air samples [148–152]. Impingers or gas-washing bottles were successfully used to trap headspace volatiles formed from heated pork fat [63]. In this study, cysteamine solutions were also used in impingers to trap formaldehyde (7.99 μg/L of headspace) and acetaldehyde (49.1 μg/L of headspace). In addition to the two RCCs, 17 other aldehydes were recovered as thiazolidine derivatives from the same sample.

A unique method for trapping RCCs from samples of complex matrices such as lipid-rich foods was introduced in the late 1980s [60]. Fig. 5 shows a systematic diagram of a simultaneous purging and solvent extraction apparatus (SPSE). The apparatus is a gas-washing bottle (impinger) and a liquid–liquid continuous extractor in tandem. Later this apparatus was named the simultaneous purging and solvent extraction (SPSE) apparatus. Volatile compounds formed in the headspace from a sample are purged into the water or reagent solution and simultaneously and continuously extracted with an organic solvent (dichloromethane). A total of 141 volatile compounds formed

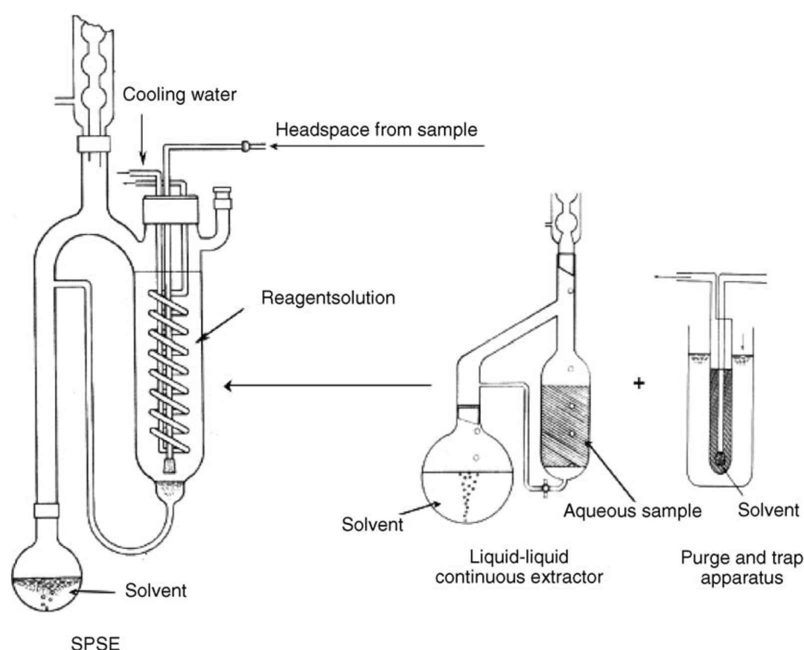

Fig. 5. Systematic diagram of a simultaneous purging and solvent extraction apparatus (SPSE).

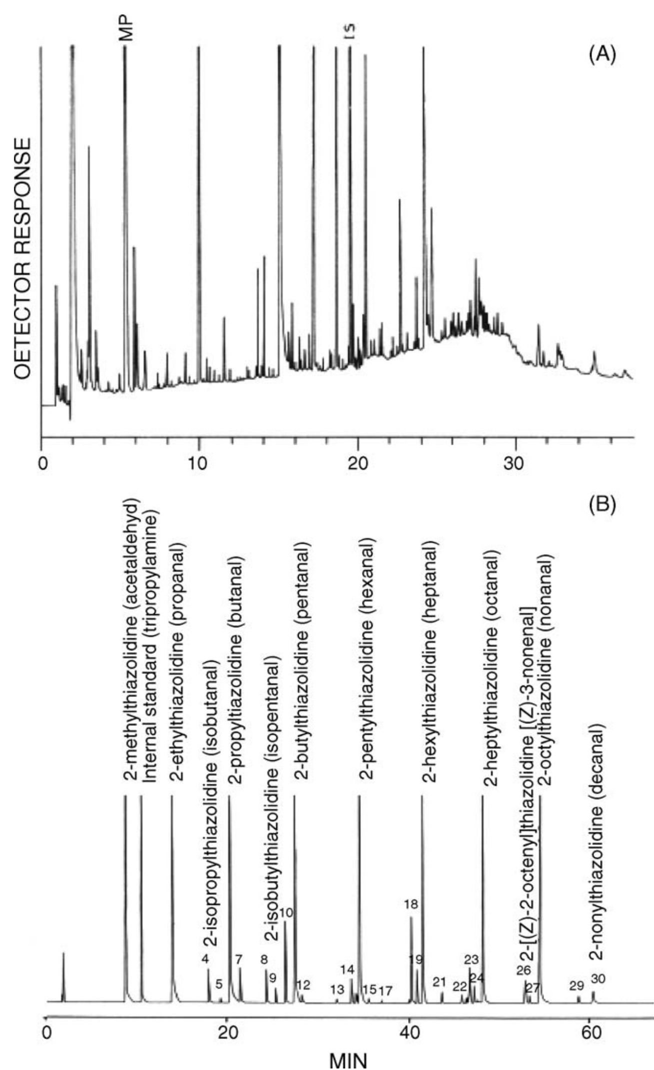

Fig. 6. GC of extracts from a water trap (A) and a cysteamine trap (B) obtained using a SPSE.

in the headspace from cooked whole egg, egg yolk, and egg white were isolated and identified by using SPSE/GC/MS [153]. In this study, water was used as a trapping solvent. Therefore, major RCCs such as formaldehyde, acrolein, and acetaldehyde were not recovered, However, if a cysteamine solution is used to trap headspace from a sample, trace levels of RCCs, including formaldehyde and acetaldehyde, can be recovered [60]. Fig. 6 shows a typical gas chromatogram of dichloromethane extract obtained from heated beef fat by using a SPSE with either a water trap (A) or a cysteamine solution trap (B).

Trace levels of formaldehyde expired from experimental mice were collected and analyzed using the apparatus shown in Fig. 7. Formaldehyde was recovered at a level of $1356 \pm 234\,\text{nmol/kg}^{0.75}$ from mice with tumors and $898 \pm 97\,\text{nmol/kg}^{0.75}$ from mice without tumors. The results suggest that monitoring the amount of formaldehyde in expired air may enable researchers to detect the presence of tumors without sacrificing animals [135]. Table 3 shows the results of formaldehyde and acetaldehyde analyses in various samples.

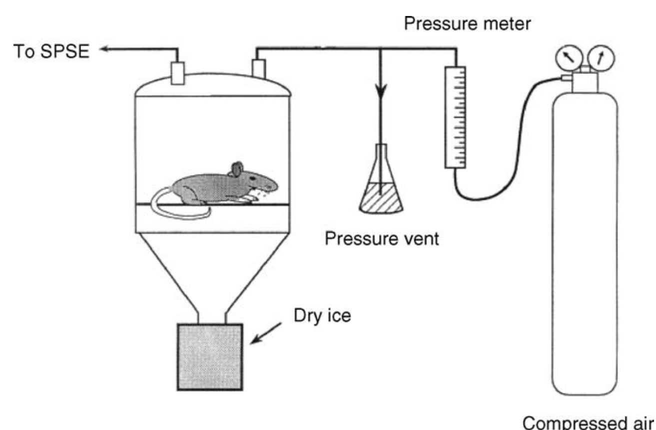

Fig. 7. Systematic diagram of an apparatus prepared to trap expired air from mice.

4.5. Application of N-methyl hydrazine derivatives to α,β-unsaturated aldehydes analysis

Acrolein (α,β-unsaturated aldehyde) does not react with cysteamime. However, direct analysis of acrolein is also extremely difficult for the reasons mentioned above. In addition, acrolein tends to polymerize readily in an aqueous solution [154]. It is, therefore, necessary to prepare an appropriate derivative for trace analysis of acrolein. Acrolein formed in thermally oxidized cooking oils was analyzed after being derivatized to 3-morpholinopropanal with morpholine [61]. One drawback of this derivative is that its analysis must be performed shortly after derivatization because it is polymerized within a short time. Later, a more stable derivative, 1-methyl-2-pyrazoline, was prepared with *N*-methyl hydrazine. Fig. 8 illustrates the general reactions between *N*-methyl hydrazine and α,β-unsaturated aldehydes or β-dicarbonyl compounds. Acrolein and another α,β-unsaturated lipid peroxidation product, 4-HN [155], were detected using this derivatizing agent. Acrolein formed in headspace from thermally oxidized oils was trapped in a dichloromethane solution of *N*-methyl hydrazine, and then analyzed with GC/NPD. The detection limit of acrolein as 1-methyl-1-methylpyrazoline was 5.9 pg in this method [156]. 4-HN is not classified as an RCC but it has been known as one of the major lipid peroxidation products [155]. 4-HN was, therefore, analyzed as one of the RCCs formed in oxidized lipids by using *N*-methyl hydrazine derivative [68]. Table 4 shows the results of acrolein, 4-HN, and MA analyses.

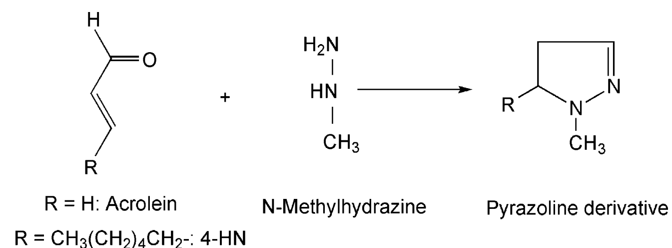

Fig. 8. Formation of pyrazoline dierivatives from α,β-unsaturated aldehydes.

Table 4
Amounts of acrolein, 4-HN, and MA found in various samples analyzed using *N*-methyl hydrazine derivatives

Sample	Oxidation method	Amount	Ref.
Acrolein			
Arachidonic acid	Fenton	7.7 nmol/mg acid	[68]
Ethyl arachidonate	Fenton	13.3 nmol/mg ester	[68]
Linolenic acid	Fenton	4.0 nmol/mg acid	[68]
Ethyl linolenate	Fenton	8.2 nmol/mg ester	[68]
Linoleic acid	Fenton	9.3 nmol/mg acid	[68]
Ethyl linoleate	Fenton	0.8 nmol/mg ester	[68]
Arachidonic acid	UV	2.5 μol/mg acid	[54]
Linolenic acid	UV	0.36 μg/mg acid	[54]
Cod liver oil	UV	1.65–10.9 nmol/mg oil	[55]
Triolein	UV	0.02–1.05 nmol/mg triolein	[57]
Lard	Heat	109 μg/L headspace	[63]
Corn oil	Heat	164 μg/L headspace	[62]
Sunflower oil	Heat	163 μg/L headspace	[62]
Cotton seed oil	Heat	5.16 μg/L headspace	[62]
Kitchen air	–	0.21–2.96 μg/L air	[156]
Cigarette smoke	–	124.4–337.4 μg/cigarette	[139]
Cod liver oil	Fenton	61.2 nmol/mL oil	[157]
Cigarette smoke	–	220–468 μg/cigarette	[101]
4-HN			
Arachidonic acid	UV	0.17 μg/mg acid	[54]
Linoleic acid	UV	0.09 μg/mg acid	[54]
Linolenic acid	UV	0.07 μg/mg acid	[54]
Arachidonic acid	Fenton	10.3 nmol/mg acid	[68]
Ethyl arachidonate	Fenton	26.1 nmol/mg acid	[68]
Linoleic acid	Fenton	15.1 nmol/mg acid	[68]
Ethyl linoleate	Fenton	9.1 nmol/mg acid	[68]
Cod liver oil	Fenton	6.83 nmol/mL oil	[157]
MA			
Cod liver oil	UV	3.8–190.2 nmol/mg oil	[55]
Ethyl arachidonate	Microwave	2.3–11.2 nmol/mg ester	[134]
Ethyl linolenate	Microwave	1.3–5.3 nmol/mg ester	[134]
Ethyl linoleate	Microwave	0.2–1.7 nmol/mg ester	[134]
Ethyl arachidonate	Heat	2.3–10.8 nmol/mg ester	[134]
Ethyl linolenate	Heat	1.3–5.3 nmol/mg ester	[134]
Ethyl linoleate	Heat	0.2–1.2 nmol/mg ester	[134]
Squalene	UV	0.73 nmol/mg squalene	[53]
Rat liver	CCl_4	0.78–1.39 μg/g liver	[158]
Squalene	UV	0.17–1.10 nmol/μmol squalene	[52]
Corn oil	UV	8.52–56.24 μg/g oil	[51]
Beef fat	UV	5.99–25.01 μg/g fat	[51]
Spleen (mice)	–	0.63 (control)–2.97 nmol/g bw (tumor bearing)	[145]
Plasma (mice)	–	23.4 nmol/g plasma	[145]
Red blood cell (mice)	–	47.2 nmol/g cell	[145]
Animal liver	CCl_4	0.06 (dog)–0.36 μg/mg protein (rat)	[159]
Cod liver oil	Fenton	60 nmol/μg oil	[157]
Ethyl arachidonate	Fenton	30 nmol/μg ester	[157]
Ethyl linolenate	Fenton	17.5 nmol/μg ester	[157]
Ethyl linoleate	Fenton	15.0 nmol/μg ester	[157]
2′ Deoxy guanosine	Fenton	213 nmol/16 μg guanosine	[160]
2′-Deoxy cytidine	Fenton	130.6 nmol/μg cytidine	[160]
2′-Deoxy adenosine	Fenton	85.06 nmol/μg adenosine	[160]
Thymidine	Fenton	84.46 nmol/μg thymidine	[160]
DNA (calf thymus)	Fenton	4.0–12.8 nmol/mg DNA	[161]
Fish oil	Heat	157 (tuna)–1070 ppm (salmon)	[65]
Vegetable oil	Heat	6.65 (corn)–35.9 ppm (soybean)	[160]
Arachidonic acid	Fenton	4.8–27.5 nmol/mg acid	[68]
Ethyl arachidonate	Fenton	13.0–97.2 nmol/mg ester	[68]
Linoleic acid	Fenton	0.7–57.9 nmol/mg acid	[68]
Ethyl linoleate	Fenton	0.1–43.1 nmol/mg ester	[68]
Arachdonic acid	UV	0.74–5.7 μg/mg acid	[54]
Linoleic acid	UV	0.06–0.16 μg/mg acid	[54]
Linolenic acid	UV	0.46–2.41 μg/mg acid	[54]
Squalene	UV	0–0.06 μg/mg squalene	[54]
Probucol	Fenton	103.9–2616.5 nmol/3 μmol probucol	[162]
Cigarette smoke	–	18.9–36.0 μg/cigarette	[101]

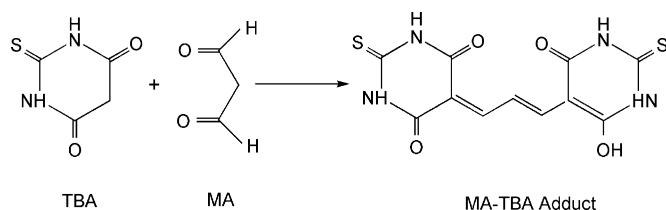

Fig. 9. Formation of MA-TBA adducts from TBA and MA.

4.6. Conventional method for MA determination

As mentioned above, MA is most widely used as a biomarker of lipid peroxidation [77,163] associated with various diseases. Among MA assays, the thiobarbituric acid (TBA) assay has been the most commonly used. In the late 1950s, estimation of 2-deoxy sugars was conducted using the MA-TBA reaction and a colorimetric method [164,165]. Fig. 9 shows a MA-TBA adduct which has a UV absorption at 535 nm. Later, the MA-TBA adduct was measured by a spectrophotometer to assess antioxidant effectiveness in pharmaceutical oils [166]. Consequently, the MA-TBA assay became the most popular method for studies related to lipid peroxidation. However, it was found that TBA reacts with many different carbonyl compounds formed from lipid peroxidation and their TBA adducts absorb the same UV wavelength as is absorbed by the MA-TBA adduct. Therefore, the TBA assay is not specific to MA, although it is still a convenient method for assessing lipid peroxidation and continues to be widely used. Later, total RCCs reacted with TBA came to be called TBA reacting substances (TBARS). The specific determination of the MA-TBA adduct formed in biological samples, such as serum and plasma, upon lipid peroxidation was performed using HPLC [167,168]. As mentioned above, the sensitivity of HPLC is not as high as that of GC, but the MA-TBA adduct is not applicable to GC due to its low volatility. However, the recent development of LC/MS can achieve a higher efficiency of HPLC analysis on TBARS [169,170].

4.7. Application of *N*-methyl hydrazine derivative to MA analysis

The *N*-methyl hydrazine derivative of MA (Fig. 10), 1-methyl pyrazole is an ideal chemical to be analyzed by a GC/NPD because it is reasonably volatile and contains two nitrogen atoms. MA reacts readily with *N*-methyl hydrazine under mild conditions (at room temperature and pH 7). Trace levels of MA were successfully detected in various matrices associated with lipid peroxidation, including fatty acids [54,68,70],

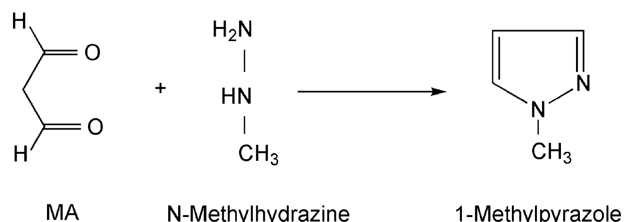

Fig. 10. Formation of 1-methylpyrazole from MA and *N*-methyl hydrazine.

cod liver oil [55,157], dietary oils [65], and biological samples from animals [145,159]. For example, cod liver oil produced 3.8–190.2 nmol/mg of MA upon UV irradiation [55]. Microwave irradiation was proved to induce lipid peroxidation in a study involving the analysis of MA formed from fatty acid ethyl esters [134].

4.8. Antioxidant studies on lipid peroxidation using *N*-methyl hydrazine derivative for MA analysis

The MA/GC/NPD method has been applied to the investigation of the antioxidative activities of many chemical(s) because of its high selectivity and sensitivity as shown in Table 5. Studies have covered a wide range of substances, from pure chemicals such as vitamins [142,177], flavonoids [100,172,181], and volatile chemicals, to extracts from natural plants [171,173,174,182,183]. Detailed review of these antioxidant studies is not within the scope of this review.

Table 5
Antioxidant studies performed using MA as a biomarker

Chemical(s) tested	Testing system	Ref.
Volatiles from Eucalyptus	Cod liver oil/Fenton	[171]
Flavonoids, phenolic acids	Ethyl arachidonate/Fenton	[172]
Aroma extract of clove bud	Cod liver oil/Fenton	[173]
Aroma components from clove and eucalyptus	Blood plasma/Fenton	[174]
Flavonoid from barley leaves, vitamin C, DMHF[a], pubucol[b]	Blood plasma/Fenton	[142]
Ferulic acid, saponarin	LDL/Fenton	[175]
Lacinilene A, naringin, galangin, rutin	Ethyl linoleate/Fenton	[176]
Lacinilene A, naringin, galangin, rutin	Ethyl linolenate/Fenton	[176]
Lacinilene A, naringin, galangin, rutin	Ethyl arachidonate/Fenton	[176]
α-Tocopherol, β-carotene, saponarin	ω-3 Fatty acids/Fenton	[177]
Aroma extracts from rosemary and sage	Ground beef/heat	[178]
Physodic acid (lichen constituent)	Rat liver microsome/CCl_4	[159]
Physodic acid (lichen constituent)	Monkey liver microsome/CCl_4	[159]
Flavonoid from barley leaves	Cod liver oil/Fenton	[100]
Flavonoid from barley leaves	Lecithin I or II/Fenton	[100]
Flavonoid from barley leaves	Squalene/UV	[178]
Flavonoid from barley leaves	Ethyl linoleate/UV	[178]
Flavonoid from barley leaves	Ethyl linoleate/Fenton	[178]
Flavonoid from barley leaves	Ethyl linolenate/Fenton	[178]
Flavonoid from barley leaves	Ethy arachidonate/Fenton	[178]
EDTA, Ferulic acid, BHT, vitamin E	Ethyl linoleate/Fenton	[179]
EDTA, Ferulic acid, BHT, vitamin E	Rat liver microsome/ADP/$FeSO_4$	[180]
Flavonoids, anthocyanins	Calf thymus DNA/Fenton	[181]
Volatiles from beans	Blood plasma/Fenton	[182]
Volatiles from beechwood creosote	Ethyl arachidonate/Fenton	[183]

[a] 2,5-Dimethyl-4-hydroxy-3(2H)-furanone.
[b] 4,4′-(Isopropylidenedithio)bis(2,6-di-*tert*-butylphenol).

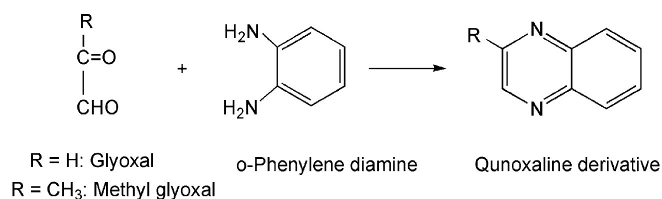

Fig. 11. Formation of quinoxaline derivatives from glyoxal or methyl glyoxal and *o*-phenylene diamine.

4.9. Application of o-phenylene diamine derivatives to α-dicarbonyl compounds

Glyoxal and methyl glyoxal have not received much attention as a lipid peroxidation product compared with MA. However, as noted previously, they are important products due to their toxicity [94–97]. It is necessary to prepare stable and less-water-soluble derivatives in order to be able to analyze these compounds by GC. In a study focused on the analysis of formaldehyde as a thiazolidine derivative, it was found that methyl glyoxal reacts with cysteamine to give 2-acetyl thiazolidine [131]. Among 17 foods and beverages analyzed by using this derivative, coffee samples were found to contain the highest levels of methyl glyoxal: 23 ppm in instant; 25 ppm in brewed regular; and 47 ppm in brewed decaffeinated [131]. One drawback of this method is that the yield of 2-acetyl thiazolidine was influenced by the presence of other carbonyl compounds such as D-glucose [131]. Later, the more selective reagent *o*-phenylene diamine was used to prepare better derivatives (quinoxalines) for α-dicarbonyl compounds including glyoxal, methyl glyoxal, and diacetyl [56]. Fig. 11 shows the reaction scheme of α-dicarbonyl compounds and *o*-phenylene diamine. Squalene was found to form glyoxal (1.22–9.59 nmol/mg) and methyl glyoxal (2.45–14.41 nmol/mg) upon UV irradiation by this method [56]. Later, this method was used to detect glyoxal and methyl glyoxal formed in thermally oxidized dietary oils [65]. Fig. 12 shows the results of this study. α-Dicarbonyl compounds found in cigarette smoke as *o*-phenylene diamine derivatives were glyoxal (1.95–6.98 μg/cigarette), methyl glyoxal (13.4–59.6 μg/cigarette), and diacetyl (301–433 μg/cigarette) [101]. Table 6 shows the results of glyoxal and methyl glyoxal analyses using *o*-phenylene diamine derivatives in various samples.

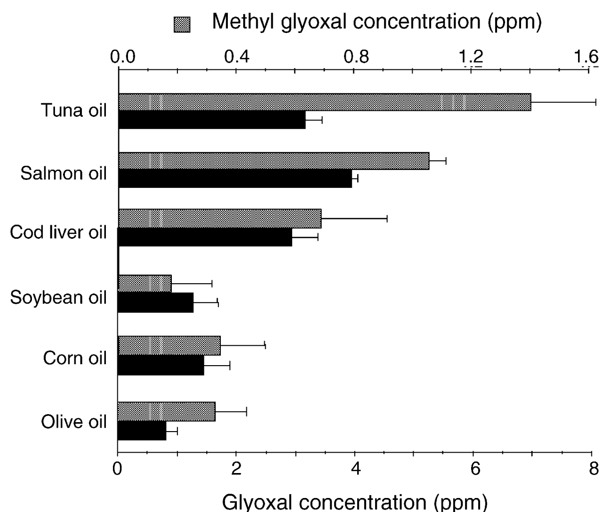

Fig. 12. Amounts of glyoxal and methyl glyoxal formed from dietary oils upon lipid peroxidation.

Table 6
Amounts of glyoxal and methyl glyoxal found in various samples analyzed using *o*-phenylene diamine derivatives

Sample	Method oxidation	Amount	Ref.
Glyoxal			
Squalene	UV	1.22-9.59 nmol/mg squalene	[56]
Cod liver oil	Fenton	11.5 nmol/10 μL oil	[100]
Lecithin I	Fenton	9.3 nmol/6.4 μmol lecithin I	[100]
Lecithin II	Fenton	9.3 nmol/6.4 μmol lecithin II	[100]
Dietary oils	Heat	0.8 (olive)–14.0 ppm (salmon)	[65]
Cigarette smoke	–	1.93–6.98 μg/cigarette	[101]
Methyl glyoxal			
Brewed regular coffee[a]	Heat	25 ppm	[131]
DECAF brewed coffee[a]	Heat	47 ppm	[131]
Cocoa[a]	Heat	1.2 ppm	[131]
Instant tea[a]	Heat	2.4 ppm	[131]
Squalene	UV	2.42–14.41 nmol/mg squalene	[56]
Dietary oils	Heat	0.03 (soybean)–2.92 ppm (tuna)	[65]
Cigarette smoke	–	13.4–59.6 μg/cigarette	[101]

[a] Cysteamine derivative was used.

5. Conclusions

Research on lipid peroxidation will continue to be an important subject to pursue from various viewpoints, including the explication and prevention of disease caused by oxidation, investigations of food and beverage deterioration, and the search for biologically active plant components such as anti-carcinogens, anti-mutagens, and antioxidants. Use of a biomarker to investigate these subjects will also remain one of the most effective techniques. Therefore, the establishment of appropriate analytical methods for biomarkers suited to investigating lipid peroxidation associated with the various aspects of lipid peroxidation listed above is critical. RCCs might remain as a biomarker for lipid peroxidation. Formaldehyde and acetaldehyde are important lipid peroxidation products, as well as toxic contaminants in foods and in the environment. MA will continued to be used as a biomarker of lipid peroxidation. The TBA assay is a simple and convenient method for monitoring lipid peroxidation, and significant developments in LC/MS technology may promote the use of the TBA assay to measure specific amounts of the MA-TBA adduct more accurately. However, use of GC should still continue to be one of the most significant methods used to monitor lipid peroxidation because of its high sensitivity, selectivity, and resolution. Sample preparation steps, such as recovery of RCCs from lipids for GC analysis have also improved tremendously.

For example, the recent development of the solid phase extraction method affords the possibility of isolating RCCs from lipid samples at trace levels. Finally, continuing development of GC, LC, GC/MS, and LC/MS is expected, and research on determination of RCCs formed from lipid peroxidation will continue to advance.

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