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Thiobarbituric acid test for monitoring lipid oxidation in meat

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Lipid oxidation is a significant problem relative to off-flavour and off-odour, and warmed-over flavour appears to be related to lipid oxidation in meat. In this review article the thiobarbituric acid (TBA) test for lipid oxidation measurements is described. Works on other techniques to measure lipid oxidation, as determination of malondialdehyde content by high performance liquid chromatography or gas chromatography are reviewed. A detailed description of the different TBA test procedures, possible interferences and limitations of this technique are also given. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

One of the most important causes of meat food deterioration is lipid oxidation, which affects fatty acids, particularly polyunsaturated fatty acids. (Gray, 1978; Allen & Allen, 1981; Pearson *et al.*, 1983; Fennema, 1993).

This lipid autooxidative degradation gives products that change the food quality, e.g. the colour, aroma, flavour, texture and even the nutritive value. (Eriksson, 1982; Love, 1983; Fennema, 1993).

This modification of fatty acids is principally carried out by an autocatalytic mechanism of 'free radicals', called autooxidation, consisting of three phases (Gray, 1978; Allen & Hamilton, 1983; Raharjo & Sofos, 1993):

1. Initiation:
   (a) $RH + O_2 \rightarrow R\cdot + \cdot OOH$
2. Propagation:
   (b) $R\cdot + O_2 \rightarrow ROO\cdot$
   (c) $RH + ROO\cdot \rightarrow ROOH + R\cdot$
   (d) $ROOH \rightarrow RO\cdot + \cdot OH$
3. Termination:
   (e) $R\cdot + R\cdot \rightarrow R-R$
   (f) $R\cdot + ROO\cdot \rightarrow ROOR$
   (g) $ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$

Hydroperoxides (ROOH) are considered to be the most important initial reaction products that are obtained from lipid oxidation; they are a labile species, of very transitory nature, which undergo changes and deterioration with the radicals. Their breakage causes secondary products such as pentanal, hexanal, 4-hydroxynonenal and malondialdehyde (MDA) (Pearson *et al.*, 1983; Raharjo & Sofos, 1993).

MALONDIALDEHYDE (AND OTHER TBA-REACTIVE SUBSTANCES)

MDA is a three-carbon dialdehyde with carbonyl groups at the C-1 and C-3 positions (Fig. 1). There are different theories about the possible mechanisms of MDA formation, through hydroperoxides formed from polyunsaturated fatty acids, with three double bonds (triene) or more, associated with phospholipids, their primary substrates in animal food. Dahle *et al.* (1962) postulated a mechanism for the formation of MDA, based on investigations which showed that only peroxides which possessed α or β unsaturations to the peroxide group, were capable of undergoing cyclization to finally form MDA.

However, some studies point to the presence of small amounts of MDA from:

(a) —fatty acids with less than three double bonds (Tarladgis *et al.*, 1960; Pryor *et al.*, 1976); in this case, MDA production is partially due to the secondary oxidation of primary carbonyl compounds (e.g. 2-nonenal) (Sinnhuber & Yu, 1977);
(b) —endoperoxides involved in the synthesis of prostaglandins, which could be non-volatile MDA precursors capable of yielding MDA with heat or acids (Pryor *et al.*, 1976);

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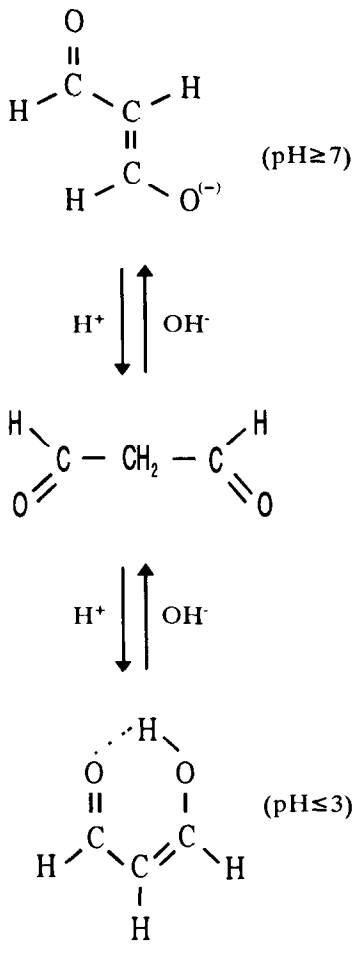

Fig. 1. Structural changes of malondialdehyde with the pH.

(c) —iron-dependent oxidative degradation of amino acids (aa), complex carbohydrates, pentoses and hexoses and from free radical products produced by gamma-irradiation treatments '*in vivo*' (Bird & Draper, 1984). The amounts of the MDA-like materials produced depend on the energy of irradiation (Dennis & Shibamoto, 1989; Yeo & Shibamoto, 1992).

The substances that react with TBA but which are not MDA, are called TBA-reactive substances (TBARS) because:

(a) they react with TBA to give an adduct whose spectrum is identical with that obtained from MDA standard;
(b) their UV spectrum is identical with that of MDA standard at both pH 7·0 and 2·0;
(c) they coelute with MDA standard when are analyzed by HPLC (Cheeseman *et al.*, 1988);
(d) the yields of MDA determined by the TBA test agree with those determined by the HPLC method.

Polyunsaturated fatty acids in meats are more likely to be found in esterified form with phospholipids than with triglycerides (Igene *et al.*, 1980; Pikul & Kummerow, 1991). Therefore, the phospholipid fractions have been identified as primary substrates in the development of oxidative deterioration in muscle foods (Igene *et al.*, 1979, 1980; Melton, 1983). A study by Pikul and Kummerow (1991) revealed that relatively high levels of arachidonic acid as well as dodecatetraenoic, dodecapentaenoic and dodecahexaenoic acids are found in phosphatidylserine, a phosphatidylchloline. These phospholipids were responsible for generation of many of the TBARS, including MDA in chicken liver, heart, plasma and egg (Pikul & Kummerow, 1991).

Factors which determine the extent and amount of MDA formation from peroxidized polyunsaturated fatty acids are: the degree of fatty acid unsaturation (Dahle *et al.*, 1962; Pryor *et al.*, 1976); the presence of metals (Janero & Burghardt, 1989); pH (Chen & Waimaleongora-Ek, 1981); and the temperature and duration of heating (Pikul *et al.*, 1984). Iron catalyzes fatty acid hydroperoxide decomposition to MDA at physiological pH and temperature (Janero & Burghardt, 1989). These studies suggest that the degradation products of fatty acid hydroperoxides in living and post-mortem tissues may differ from the degradation products obtained by heat and acid treatment during the TBA test.

MDA is thought to be a carcinogenic initiator and mutagen, and therefore can affect the safety of food. It has been found that the type of cooking (e.g. microwaves, roasting, etc.), time and temperature affected MDA content (Igene *et al.*, 1979).

MDA can complex with aminoacids, proteins, glycogen and other food constituents to form products in which MDA is in a bound form (Kwon *et al.*, 1965).

The principal interactions are:

(a) *Interaction of MDA with water*: MDA occurs mainly as its enolic tautomer in aqueous solution. Dissociation of the enolic aldehyde starts at pH 2·8 and is complete at pH 6·5. (Mashio & Kimura, 1960; Kwon & Watts, 1963; Saunders & May, 1963). MDA exists completely as a volatile specie only below pH 2·8. Volatility is attributable to intramolecular hydrogen bond formation. Above pH 6·5, no volatilization of the aldehyde occurred. At this pH, the aldehyde is totally dissociated into its anion (Kwon *et al.*, 1965).
(b) *Interaction of MDA with proteins*: MDA can be bound to the water soluble protein fractions, aldolase and myosin (1·3–2·6 g MDA/mg protein), and both acidification and heating are necessary to volatilize MDA from these protein complexes (Kwon *et al.*, 1965). MDA reacts preferentially with histidine, arginine, tyrosine and methionine.
(c) *Interaction of MDA with carbohydrates*: only MDA reaction with glycogen could be demostrated (the amount of MDA bound per mg of glycogen was comparable to that bound by proteins). The difference of reactivity between

glycogen and starch suggest that this binding requires a certain type of organized structure.

It is difficult to determine the optimal conditions for the release of MDA from its bound forms, which differ from one material to another and require different conditions for hydrolysis. It is also difficult to hydrolyze all of the MDA bound to meat protein without using strong acidic conditions and heating (Kwon *et al.*, 1965) which endanger the stability of the MDA–TBA complex (Draper *et al.*, 1986) and cause problems related to the quantitative recovery of MDA during the TBA test.

LIPID OXIDATION MEASUREMENT TECHNIQUES

Numerous analytical procedures for the measurement of lipid oxidation in meats and meat products have been described. There are chemical methods such as: peroxide value, Kreis test, total and volatile carbonyl compounds (Henick *et al.*, 1954), TBA test; and physical methods such as: polarography (Lewis *et al.*, 1949), infrared spectroscopy (Henick, 1951; Ahlers & McTaggart, 1954), refractometry (Arya *et al.*, 1969), fluorescence (Dillard & Tappel, 1971, 1973) and conjugated diene method (Parr & Swoboda, 1976).

Currently, the techniques most widely used to measure lipid oxidation are:

1. *Determination of the peroxide value*: this method is limited by the transitory nature of the peroxides which are intermediate products in the formation of carbonyl compounds. Numerous analytical procedures for the measurement of the peroxide value are described in the literature: iodometric method (Lea, 1931; Wheeler, 1932) and colorimetric method (Lips *et al.*, 1943). However, some investigators showed that the rancidity of animal feeds correlated better (rs = 0·92) with MDA content than peroxide value. When the TBA test is compared to peroxide value, several contradictory reports are found; Dahle *et al.* (1962) reported a linear relationship between TBA value and peroxide value for polyunsaturated fatty acids. Gray (1978) concluded that TBA value correlates well with peroxide value only in oils containing fatty acids with three or more double bonds. The TBA value and peroxide number in frozen cooked pork meat was compared by Younathan and Watts (1960), who reported that while TBA values increase rapidly following cooking, exceeding the point of acceptability (threshold for sensory perception: TBA value = 5, peroxide m.e.q./100 g fat = 20), peroxide numbers were still low.
2. Determination of hexanal content (Shahidi *et al.*, 1987): this involves measuring the hexanal content of a steam distillate and subsequently submitting it to gas chromatographic analysis. These authors found, in general, that the hexanal amount present in the distillates showed linear correlation with the corresponding TBA values.
3. Determination of MDA content: the relation between MDA content and lipid oxidation has aroused the interest of investigators to search for techniques that permit it to be followed and quantified, and so different analytical techniques have been described in the literature.

MDA determination by ultraviolet spectrophotometry (Kwon & Watts, 1963)

This method uses the absorbance difference between acidified and basified MDA solutions at 267 nm. The ultraviolet absorption spectra of MDA is pH-dependent, and their absorption in this region between pH 3·0 and 7·0 shifts progressively. This behaviour is attributed to the progressive dissociation of the enolic hydrogen with increasing pH: at pH 3 or lower, the compound is thought to have a cyclic planar *δ-cis*-configuration, with an intramolecular hidrogen bond; above pH 7·0 it is completely dissociated and exists as a planar *δ-trans*-enolate anion (Fig. 1). The method has been successfully applied to the assay of MDA in distillates from rancid foods. Its sensitivity is only about 40% of the TBA test, but this is sufficient to detect threshold levels of rancidity. The test is simpler, much more rapid, and more specific than the TBA test.

Determination of MDA by HPLC

The first reference about the use of this method were by Kakuda *et al.* (1981). They used it for the quantification of MDA in aqueous distillated and found a linear correlation between TBA values and HPLC results. Later it was used by Williams *et al.* (1983) to determine meat oxidation. Other investigators developed an HPLC method for the direct analysis of MDA with no previous distillation step (Esterbauer & Slater, 1981; Bird *et al.*, 1983; Csallany *et al.*, 1984).

Gas chromatography

The MDA determination with this method requires its previous derivatization. Several conditions for MDA derivatization have been proposed: 2-hydroxypyrimidine (Hamberg *et al.*, 1968), 2,3-propanediol (Lakshminarayana & Cornwell, 1986), 2-hydrazinobenzothiazole (Beljean-Leymarie & Bruna, 1988), pentafluoro-phenylhydrazine (Tomita *et al.*, 1990), etc. Depending on the method, either free MDA or bound and free MDA may be determined.

TBA test

This method is based on the MDA reaction with TBA to obtain a red pigment, which results from the con-

densation of two molecules of TBA with one molecule of MDA and the probable elimination of two molecules of water (Sinnhuber *et al.*, 1958).

TBA TEST

The TBA test for the measurement of lipid oxidation was proposed over 40 years ago. Kohn and Liversedge (1944) observed that animal tissues which have been incubated aerobically with TBA produced a pink colour. Bernheim *et al.* (1948) found that the colour was the result of a complex formed from oxidation products of unsaturated fatty compounds and TBA. Yu and Sinnhuber (1957) reported that MDA, a product of lipid oxidation, was the major TBA reactive substance although later other investigators reported that other oxidation products may also be involved, including α,β-unsaturated aldehydes (e.g. 4-hydroxyalkenals) and several unidentified non-volatile precursors of these substances. Tarladgis *et al.* (1960) described the distillation method as a technique to measure MDA content and, later, many modifications and new methods were described.

Nature of the reaction

The reaction with TBA occurs by attack of the monoenolic form of MDA on the active methylene groups of TBA (Fig. 2). Visible and ultraviolet spectrophotometry of the pigment confirms the primary maximum at 532–535 nm and a secondary one at 245–305 nm (Sinnhuber *et al.*, 1958).

The intensity of colour is a measure of MDA concentration (Tarladgis *et al.*, 1960, 1964) and has been organoleptically correlated with the rancidity (Zipser *et al.*, 1964) and W.O.F. (Igene & Pearson, 1979; Igene *et al.*, 1985). This TBA test has been widely used as a measure of oxidative rancidity in fat foods, particularly in meat products. It compares the absorbance of a MDA–TBA complex with a standard made from 1,1,3,3 tetraethoxypropane (TEP) or 1,1,3,3 tetramethoxypropane (TMP), because the MDA can be obtained by acid hydrolysis from TMP or TEP in an equimolecular reaction. The speed of the reaction of TBA with MDA depends on the concentration of the TBA-solution, temperature and pH. Raharjo *et al.* (1992) reported that by increasing the concentration of TBA solution from 20 to 80 mM, the TBA reaction time at 94 ± 1°C decreased from 30 min to approximately 5 min. The maximun intensity of the red MDA–TBA complex could be obtained in 60 min in a boiling water bath or in 15 h when the reaction proceeded at room temperature (Tarladgis *et al.*, 1964). The speed of the reaction was faster when the pH of the medium was 3 or lower (Kwon & Watts, 1963).

Different TBA test procedures

Different ways of assaying the TBA test in meat or meat products have been reported:

(a) Direct reaction of TBA solution on the food products and extraction of the red pigment produced (Turner *et al.*, 1954; Sinnhuber & Yu, 1958; Pokorny & Dieffenbacher, 1989).
(b) Aqueous acid extraction of meat sample (Patton & Kurtz, 1951; Tarladgis *et al.*, 1964; Vyncke, 1975; Rosmini *et al.*, 1994).
(c) Reaction on a portion of the steam distillate of meat samples (Tarladgis & Watts, 1960; Rhee & Watts, 1966; Pikul *et al.*, 1983, 1989; Shahidi *et al.*, 1987).
(d) Reaction on a previously extracted lipid portion of meat samples (Younathan & Watts, 1960; Pikul *et al.*, 1983, 1989).
(e) Spectrofluorometric method: this method uses the fluorescence properties of TBA–MDA complex to measure the MDA levels in the sample (Williams *et al.*, 1983; Yagi, 1984, 1989).

Direct reaction on whole sample

This consists of directly heating the meat sample with TBA solution under acidic conditions and extraction of the red pigment with butanol. It is a quantitative method but is very time consuming and involves many solvent extractions (Yu & Sinnhuber, 1957; Sinnhuber & Yu, 1958; Almandos *et al.*, 1986). It is also thought that oxidation is brought on by the nature of the whole sample test itself (Tarladgis *et al.*, 1960).

The distillation method

This consists of a reaction between TBA solution with a portion of distillate; this has been found to give lower

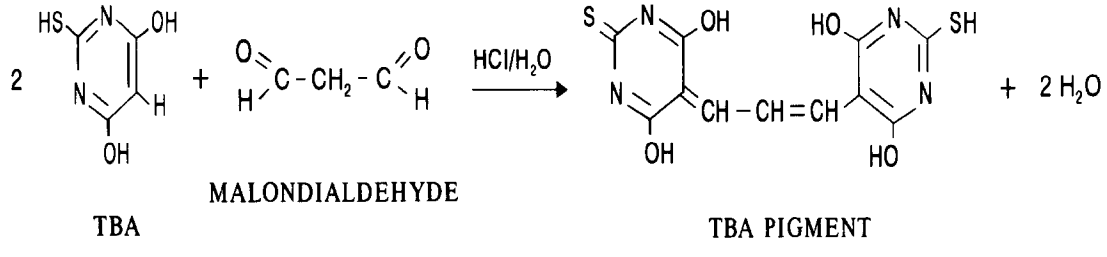

Fig. 2. Reaction between TBA and MDA to form the TBA pigment.

recoveries compared to the solvent extraction method (Siu & Draper, 1978; Williams *et al.*, 1983; Salih *et al.*, 1987) but it is considered more sensitive and also more suitable for high fat samples (> 10%) where turbidity may occur in extracted samples (Siu & Draper, 1978; Williams *et al.*, 1983; Salih *et al.*, 1987).

The extraction method

This is based on an aqueous acid extraction of the sample (with trichloroacetic acid) prior to reaction with TBA. This may be considered as the best method for estimating the MDA content in meat samples, because the meat itself is not exposed to heat treatment (Witte *et al.*, 1970). It is faster and easier to perform than the distillation method and is recommended for use where a large number of samples need to be analyzed rapidly (Salih *et al.*, 1987; Pikul *et al.*, 1989). Sometimes, some impurities such as water soluble proteins (Schmedes & Holmes, 1989), peptides (Draper & Hadley, 1990) and other aldehydes (Kosugi *et al.*, 1988) may still be present in the meat extracts and this can produce interferences with the red pigment formation. Several authors reported a high correlation between the extraction and distillation methods (Witte *et al.*, 1970; Salih *et al.*, 1989; Pikul *et al.*, 1989). Witte *et al.* (1970) reported a simple regression equation ($r = 0{\cdot}845$) obtained for relating the TBA values in pork meat, by the distillation (X) and extraction methods (Y):

$$Y = 0 \cdot 0358 + 0 \cdot 3917X$$

The extraction method gives lower TBA numbers than the distillation method for duplicate samples, perhaps due to reduced sample autoxidation during the extraction process (Witte *et al.*, 1970; Vyncke, 1975; Pikul *et al.*, 1983; Salih *et al.*, 1987). This hypothesis was confirmed with later studies where no difference was reported between TBA values determined by the distillation and solvent extraction methods (Siu & Draper, 1978). Rhee (1978) attributed the higher value from the distillation method to thermal decomposition of the MDA precursor and its liberation by heat from its bound state with proteins.

The lipid extraction procedure

When this method was used, the TBA number was higher than with the distillation method (Pikul *et al.*, 1989) because the assay conditions help the oxidation. This increase in MDA recovery is produced during lipid extraction, which is usually performed by evaporation at a fairly high temperature and under a flow of nitrogen. This method was particularly appropiate when the susceptibility to oxidation of different kinds of lipids or individual lipid components (e.g. phospholipids) was studied. The advantage of this procedure is that the presence of interfering substances, such as soluble proteins peptides and pigments, in meat samples, can be eliminated.

The spectrofluorometric procedure

This is based on the fact that the excitation spectrum shows its maximun at 532 nm. This method also requires a prior lipid extraction. The specificity of the method is about the same as that of the spectrophotometric method, because other compounds originating from degradation of lipid peroxides also generate fluorescence (Shimasaki *et al.*, 1977). A recent study by Kojima *et al.* (1990) indicated that the TBA test with fluorometric detection might also detect other aldehydes of non-lipid origin.

Although tests on the whole sample or on the extracted fat may be appropriate for some samples, the distillation method has the advantage that it can be applied to any foodstuff and is both rapid and reproducible (Sidwell *et al.*, 1955). The fact that the TBARS are obtained in clear aqueous solution and that the pink reaction product can be measured accurately is a major advantage (Tarladgis *et al.*, 1960; Bird & Draper, 1984). Distillation also reduces the interferences, noted in the whole sample and extracted fat procedures, due to compounds which may be present in food samples. The main disadvantage of the distillation method is to its empirical nature, and so it requires the collection of a specified volume of distillate (Sinnhuber & Yu, 1977). However, the distillation method is the most widely used method.

Interferences

The first interferences reported in the bibliography were due to the presence of a yellow chromagen (max. 450–460 nm) overlapping the pink peak (max. 530–537 nm) causing erroneously high values if it was of sufficient intensity (Sinnhuber & Yu, 1977; Crackel *et al.*, 1988). This yellow chromagen may be formed by a variety of aldehydic compounds reacting with TBA and could be ascribed to sugars or their degradation products (Wilbur *et al.*, 1949; Turner *et al.*, 1954; Wertheim & Proctor, 1956; Baumgartner *et al.*, 1975; Salih *et al.*, 1987). These authors suggested that solvent extraction methods should only be used if compounds producing the yellow pigment are absent, or present in sufficiently small quantities not to interfere.

In the extraction method, interferences by impurities in the meat extract, such as water-soluble proteins, peptides and other aldehydes, have been described, but their involvement in the formation of the MDA–TBA red pigment can be avoided by using a reversed-phase chromatography C_{18} cartridge (Draper & Hadley, 1990; Squires, 1990). Further purification may be performed by spotting the red pigment onto C_{18} thin-layer chromatography plates, and developing with methanol–water combinations (Beckman *et al.*, 1991). Since MDA is very hydrophilic (Kishida *et al.*, 1990), most of the extractable MDA will probably be recovered by aqueous acidic extraction.

It has been reported that a product of sucrose pyrolysis appears to react with TBA to form a pink

chromagen absorbing at 532 nm, which may be evidence for the formation of a 532 nm chromagen in the total absence of lipid peroxidation (Baumgartner *et al.*, 1975).

Other interferences with numerous products, both organic (proteins, pigments, formaldehyde) and inorganic (ionic metals, Fe) have been described. None of them appears to be important and we only report the work by Bird and Draper (1984) who stated that iron salts catalyse the breakdown of hydroperoxides to MDA and catalyse degradation of amino acids, sugars (deoxyribose, hexoses, pentoses) and DNA in the presence of air to yield MDA.

Another important interference by residual nitrite has been described in cured meat products: the presence of residual nitrite in samples could produce a nitrosation of MDA. Such nitrosation renders all or a portion of the MDA unreactive in the TBA–MDA reaction thus making the TBA numbers lower than they should be. MDA reacts with nitrite in acid medium, even at room temperature (Kolodziejska *et al.*, 1990). The sulfanilamide reacts with the residual nitrite to produce a diazonium salt (Zipser & Watts, 1962) as is shown in Fig. 3. When residual nitrite is not present, or if it is present at a concentration lower than 100 ppm, the sulfanilamide added may lead to the underestimation of the TBA values because the MDA itself takes part in a competitive reaction by condensing with one or two molecules of sulfanilamide (Shahidi *et al.*, 1985; Shahidi, 1989). Kolodziejska *et al.* (1990) reported that sulfanilamide appeared to protect MDA either by decomposing nitrite or by binding MDA and subsequently making it available to the TBA reaction.

Limitations of the TBA test

The most important limitation is that MDA and other short chain carbon products of lipid oxidation are not stable for a long period of time. This is because oxidation of these products yields organic alcohols and acids, which are not determined by the TBA test (Tarladgis & Watts, 1960; Dugan, 1961; Seo, 1976; Gokalp *et al.*, 1978; Kosugi & Kikugawa, 1985; Almandos *et al.*, 1986). Kenaston *et al.* (1955) reported that the TBA test was the most sensitive of all chemical methods used for the detection of the oxidation products of linolenic and linoleic acids but was relatively insensitive to oleic acid.

Kwon and Watts (1964) found another limitation of the TBA test for dehydrated foods, because they noted that the advanced lipid oxidation still gave low TBA values; they suggested that this was due to the MDA produced being in 'volatile chelated' form, because of the absence of water, and therefore not being held in the food.

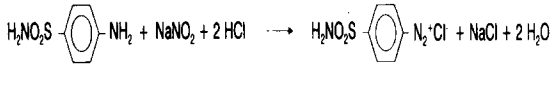

Fig. 3. Reaction between sulfanilamide and nitrite.

We can conclude that the test conditions themselves contribute to variations in the results obtained in any TBA test.

Correlations of TBA test with sensory analysis

Several authors have reported good correlations between TBA values and sensory analysis to detect rancidity in animal foods such as: milk (Dunkley & Jennings, 1951; Patton & Kurtz, 1951; Sidwell *et al.*, 1955), pork meat (Turner *et al.*, 1954), chicken meat (Salih *et al.*, 1987), and W.O.F. (Zipser *et al.*, 1964; Wilson *et al.*, 1976; Poste *et al.*, 1986) which is a flavour deterioration which develops in cooked meat but not cured, and which is related with the typical end-products of peroxidation of polyunsaturated fatty acids and phospholipids (Ruenger *et al.*, 1978; Pearson & Gray, 1983; Willemot *et al.*, 1985; St. Angelo & Bailey, 1987; Asghar *et al.*, 1988; Graf & Panter, 1991).

Melton (1985) reported that oxidized flavours were detectable at TBA numbers of 0·3–1·0 in beef or pork, 1·0 or 2·0 in chicken, and higher than 3·0 in turkey. However, these ranges of TBA numbers should not be considered as general 'reference numbers' for thresholds of rancid odour in meats, because, in addition to animal species, TBA numbers are influenced by other factors such as dietary status and age of the animals prior to slaughtering, whether the meat is raw or cooked, and the types of TBA methods used for the analyses.

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