J Amer Oil Chem Soc (2007) 84:405–411
DOI 10.1007/s11746-007-1061-5

REVIEW

Chemiluminescent Methods in Olive Oil Analysis

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Received: 1 November 2006 / Revised: 15 January 2007 / Accepted: 12 March 2007 / Published online: 18 April 2007

Abstract The present review describes chemiluminescence applications to olive oil analysis and olive mill wastewater and covers the literature published in recent years. The article has been divided into several sections that discuss specific applications for determining phenolic compounds, antioxidant activity, radical scavenging, oxidative stability and the detection of possible adulteration by seed oils. The advantages and disadvantages of the chemiluminescence detection systems for these purposes are evaluated.

Keywords Olive oil · Chemiluminescence

Introduction

Virgin olive oil is a source of healthy unsaturated fatty acids and hundreds of micronutrients, especially antioxidants, such as phenol compounds, vitamin E and carotenes [1]. The evidence indicates that the protective effects of olive oil can be ascribed not only to its high oleic acid content but also to the antioxidant properties of its polyphenols, which are absent in seed oil [2].

The quality of virgin olive oil mainly depends on the amount and the composition of the phenolic compounds in the olives when they are harvested. These compounds determine the oil's taste and its stability against oxidation, as well its nutritional and therapeutic characteristics [3]. Phenols, and in particular orthodiphenols (hydroxytyrosol, oleuropein), have been shown to make a considerable contribution to the oxidation stability of oil [4]. The level of these substances in oils is therefore essential to the evaluation of the quality of virgin olive oil, unlike edible seed oils which lose them in the various refining stages. For these reasons, qualitative and quantitative determination of these substances in oils is very important.

In principle, the analytical procedure for determining individual phenolic compounds in virgin olive oil involves three basic steps: extraction from the oil sample, analytical separation and quantification [5]. In recent years many analytical procedures have been proposed for determining the complete phenolic profile and many different methods have been applied to determine the qualitative/quantitative content of phenolic compounds in olive oil [5–9], including spectrophotometric assays (traditional) and high-resolution chromatographic techniques. Some authors have described how chemiluminescence techniques can be used to evaluate the phenolic compounds in olive oil.

Chemiluminescence (CL) is defined as the production of electromagnetic radiation (ultraviolet, visible or infrared) observed when a chemical reaction yields an electronically excited intermediate or product, which either luminesces or donates its energy to another molecule, which then luminesces [10]. Analytical methods based on chemiluminescence have become some of the leading analytical techniques because they have very good properties: sensitivity, selectivity and, in many cases, a wide linear detection range. The first often means that chemiluminescence has lower detection limits than absorption or fluorescence techniques in pursuit of the same analyte. The second, selectivity, is derived from the fact that the analyte of interest often generates its signal in the presence of normal interfering compounds which, in this case, do not

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themselves produce light when the chemiluminescent reagents are mixed together. And the third is analytically useful because samples with larger concentration ranges can be analyzed without being diluted [11]. The attractiveness of chemiluminescence as an analytical tool is the simplicity of detection: the instruments used need only provide a way of detecting light and recording the result.

To the best of our knowledge, however, there is little information available on analytical applications of chemiluminescent olive oil assay in the literature. We have reviewed the literature on chemiluminescence applications in olive oil analysis including works published up till 2006. We searched the ISI Web of Knowledge (Current Content Connect, ISI Web of Science), Scopus (Elsevier) and the SciFinder Scholar database for papers on this subject. In this paper, we have tried to reflect the current tendency to use CL techniques in this field. First, we summarize the reagents and reactions which are the basis of the CL methods applied in most of the cases described and in the Table 1, we have summarized the results obtained in the bibliographic search to olive oil analysis.

Lucigenin as a Chemiluminescent Reagent

Lucigenin (*N*,*N*′-dimethyl-9-9′-biacridinium dinitrate) is an aromatic compound and its oxidation is also a well-known chemiluminescence reaction (Fig. 1). If an aqueous lucigenin solution is mixed with a highly alkaline aqueous solution containing ethanol or acetone and an oxidant such as hydrogen peroxide or a reactive oxygen species, a very bright green emission is produced that decays to greenish blue and finally blue. The mechanism in the oxidation reaction is likely to involve a dioxetane intermediate [12]. Lucigenin reacts with hydrogen peroxide to form an unstable dioxetane which decomposes to *N*-methylacridone in an electronically excited state. The excited acridone emits light as it relaxes to a stable state. The emission can last as long as a couple of minutes under the right circumstances.

Luminophore-dependent (luminol, lucigenin, etc.) chemiluminescence is widely used to monitor the formation of reactive oxygen species (ROS) in biological and other systems. The fact that many of the phenolic compounds in olive oil react with various reactive oxygen species and affect the oxidative stability of oils and the fact that many chemiluminescent reactions are the result of a reaction with reactive oxygen species make it possible to estimate the antioxidant activity of edible oil extracts because these reactions reduce the light signal [13].

The first evidence that olive oil phenolic compounds are capable of inhibiting leukocyte 5-lipoxygenase whilst sparing the generation of prostaglandins was provided by de la Puerta et al. [14] who also showed that these compounds can reduce the generation of reactive oxygen species by intact leukocytes. The procedure involves isolating mixed peritoneal leukocytes from rats, preincubating them with test drugs and then reacting them with lucigenin.

Table 1 Summary of CL olive analysis

Chemiluminescent reactive	Study in olive oil	Reference
Lucigenin	Olive oil phenolics inhibit leukocyte 5-lipoxygenase	[14]
Lucigenin	Antioxidant activity	[13]
Lucigenin	Antioxidant activity	[15]
Luminol and cytochrome c	CL-HPLC assay quantification of hydroperoxides formed during the oxidation	[20]
Luminol and hemoglobin	Lipid peroxiradicals/ scavenging potential	[21]
Luminol and phorbol myristate acetate	Scavenging actions of hydroxytyrosol and oleuropein with respect to human neutrophils respiratory burst	[22]
Luminol and lucigenin	Reduction of free radicals in the blood	[23]
Luminol	Characterization of thermooxidative stability	[24]
Luminol and lucigenin	Antioxidant effect of hydroxytyrosol	[25]
Luminol	Determination of total antioxidant levels of olive mill wastewater	[18]
Luminol	Evaluation how ultrasonic irradiation can be used to reduce antioxidant activity of olive oil mill wastewater	[26]
Luminol	Effects of an olive oil-based lipid emulsion	[27]
TCPO	Determination of peroxide value	[28]
Rat liver microsomes	Effect of polyphenolic compounds on the non-enzymatic lipid peroxidation	[29]
Potassium superoxide in dimethylsulfoxide	Potential adulteration	[30]
18-crown-6/dimethylsulfoxide/KO_2	Antioxidant activity of genistein and oleuropein	[31]

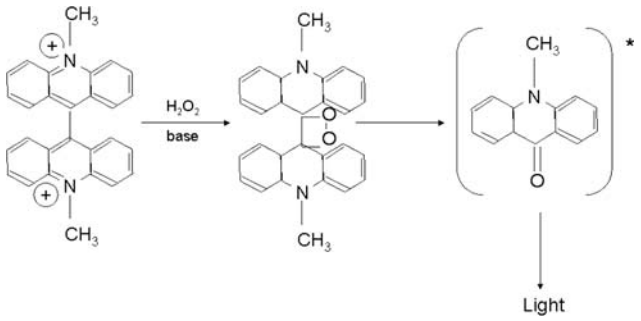

Fig. 1 CL reaction of lucigenin

These studies add the biochemical properties of olive oil components by showing that the phenolic components selectively inhibit the 5-LO (leukotriene) pathway of arachidonate metabolism in activated leukocytes but do not affect the cyclo-oxygenase pathway. As far as free radical scavenging is concerned, de la Puerta et al. have shown that oleuropein, hydroxytyrosol, tyrosol and caffeic acid can quench the superoxide chemiluminescence in phorbol myristate acetate-stimulated cells and scavenge hydrogen peroxide. The results show that the principal phenolics in the polar fraction of virgin olive oil have an array of potentially beneficial lipoxygenase inhibitory, prostaglandin-sparing, antioxidant properties.

Papadopoulos et al. [15] and Triantis et al. [13] describe a sensitive and simple procedure for measuring the antioxidant activity of commercial olive oil and seed oil aqueous methanolic extracts, which uses the chemiluminescence of lucigenin and alkaline hydrogen peroxide. All oil extracts reduce the light signals of lucigenin and it is the olive oils that show the highest antioxidant activity. The olive oils were diluted in hexane and extracted with aqueous methanol. The aqueous solutions were evaporated to dryness and the residue was taken up with aqueous methanol. To determine the total phenols in oils, extracts, phenols such caffeic acid, gallic acid, tyrosol and trolox were selected and their CL was measured at various concentrations. All these compounds showed a linear range of the light signal reduction at concentrations of 10^{-3}–10^{-2} M. The authors show that the phenol contents of oil extracts are similar to those measured and calculated in the Folin-Ciocalteu experiments. These data show that CL can be used to determine the total phenols in oils. The antioxidant activities of pure phenols were determined; caffeic acid had the highest antioxidant activity at all concentrations, followed by gallic acid and tyrosol, which is often, used as a standard for evaluating antioxidant activities. The authors show that lucigenin chemiluminescence provides the basis for a simple analytical method to estimate the antioxidant activity of aqueous extracts of olive oils.

Luminol as a Chemiluminescent Reagent

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is widely used as a chemiluminescent reagent [16]. It reacts with such oxidants as hydrogen peroxide (H_2O_2), superoxide and other reactive oxygen species in the presence of a base and a metal catalyst to produce an excited state product (3-aminophthalate) which gives off light at approximately 425 nm (Fig. 2). The intensity of the chemiluminescence is proportional to the amount of superoxide or reactive oxygen species in the sample. This superoxide reaction is an example of how chemiluminescence can be produced from luminol. In the case of luminol-induced chemiluminescence, light emission was found to increase considerably because of luminol's ability to react with hydroxyperoxide.

The literature contains numerous publications [17, 18] that show that luminol chemiluminescence is widely used as a sensitive assay to monitor free radicals and reactive metabolites. Some of the reported sources of free radicals are hydrogen peroxide and organic peroxides and it has been demonstrated that the presence of many transition metal cations [Co(II), Cu(I), Cu(II), Fe(II) and Fe(III)] catalyze the decomposition of hydrogen peroxide into hydroxyl radicals:

$$\mathrm{Co(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Co(III)} + \mathrm{HO^{\bullet}} + \mathrm{HO^{-}}$$

$$\mathrm{O_2^{\bullet}} - + \mathrm{Co(III)} \rightarrow \mathrm{O_2} + \mathrm{Co(II)}$$

The light intensity was linearly proportional to peroxide concentration. This method evaluates total antioxidant potential and total antioxidant activity [19]. The general principle is based on the ability of luminol to luminesce under the flux of free radicals. The addition of an antioxidant, which scavenges active free radicals, results in CL quenching, commonly with a pronounced induction period.

The oxidation of food lipids and edible oils causes undesirable and rancid flavours and it is important to detect

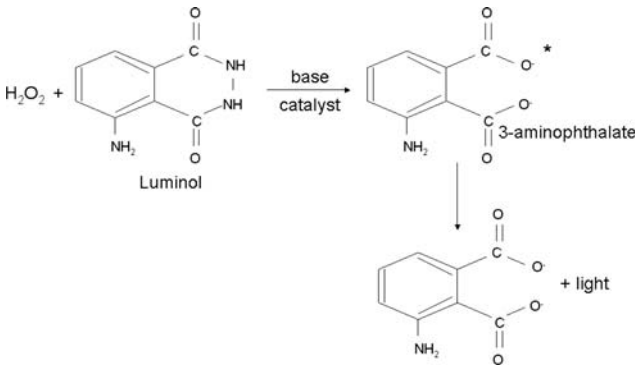

Fig. 2 CL reaction of luminol

peroxides present in the oil. Miyazawa et al. [20] describe the chemiluminescence-high performance liquid chromatography (CL-HPLC) assay of triacylglycerol hydroperoxides and how it was used to characterize the hydroperoxides formed during autoxidation and photosensitized oxidation of vegetable oils, including olive oil. The oils were allowed to autoxidize in the dark at 25 °C and the autoxidized oil, dissolved in appropriate amounts of chloroform/methanol, was analyzed by CL-HPLC (the chemiluminescence reagent consisting of cytochrome c and luminol in borate buffer). Mono-OOH oleoyl–oleoyl–linoleoylglycerol was the representative species (66% of total hydroperoxides) in olive oil. The results demonstrate that CL-HPLC is a highly sensitive tool for quantifying the molecular levels of hydroperoxides formed during the oxidation of vegetable oils.

Sawa et al. [21] have examined the possible implication of lipid peroxyl radicals ($LOO^{\bullet}$) generated from fatty acids and heme-iron in DNA damage, and hence in the possibility of colon cancer. Generation of peroxyl radicals from edible oils was quantitated by means of a luminol-enhanced chemiluminescence assay. The assay mixture contained phosphate buffer, the metal chelator diethylenetriaminepentaacetic acid (DPTA), oil and luminol. The chemiluminescence assay was started after addition of hemoglobin. Unpurified virgin and extra virgin olive oils showed no apparent generation of $LOO^{\bullet}$, even in the presence of hemoglobin. The authors also investigated the $LOO^{\bullet}$-scavenging potential of these oils by using a system generating *t*-$BuOO^{\bullet}$, based on chemiluminescence induced by *tert*-butylhydroperoxide (*t*-BuOOH) plus hemoglobin. The results showed that the extra virgin and virgin olive oils showed the highest *t*-$BuOO^{\bullet}$-scavenging potential. The authors have concluded that oils such as virgin olive oil that are rich in $LOO^{\bullet}$ scavengers are preferable not only for an anticarcinogenic potential but also for prevention of reactive oxygen-related diseases.

Visoli et al. [22] have investigated the scavenging actions of some olive oil phenolics, namely hydroxytyrosol and oleuropein, with respect to human neutrophils respiratory burst by chemiluminescence. Venous blood was collected from healthy volunteers and human neutrophils were isolated. A suspension of cells was added with luminol and placed in a luminometer. Phorbol myristate acetate (PMA) was added and the increase in chemiluminescence was recorded. The free radical production due to the respiratory burst of neutrophils, triggered by the addition of PMA was evaluated by continuous monitoring of luminol oxidation. A dose-dependent inhibition of the rate of chemiluminescence formation was observed following co-incubation of cell samples with either oleuropein or hydroxytyrosol.

Cells that exhibit respiratory bursts, such as phagocytes, produce CL. The light emitted is very weak and chemiluminogenic probes are required to increase the efficiency of the light detection. Luminol-dependent CL is thought to reflect the production of hydrogen peroxide and singlet oxygen. Yu and Tsai [23] have studied the effect of a diet enriched in monounsaturated fatty acids (olive oil) on plasma lipids. The authors have evaluated the effects of an olive oil-enriched diet plus barley leaf essence on the susceptibility of the low-density lipoprotein (LDL) subfraction to oxidation and on the whole blood free radical activity of patients with type 2 diabetes. They have found that an olive oil-enriched diet leads to increased vitamin E contents in LDL and the prolongation of the lag phase of LDL oxidation. The vitamin E content of olive oil may not be higher than that of soybean oil, but olive oil contains phenolic compounds which might protect LDL-vitamin E content. Lucigenin and luminol-amplified chemiluminescence was used to quantify superoxide radicals and oxygen free radicals in peripheral blood. The total CL counts were calculated by integrating the area under the curve and subtracting it from the background level. The results show that the addition of olive oil to the diet reduced the production of oxygen free radicals in blood. They also suggest that the radical scavenging properties of olive oil polyphenols help to decrease the level of oxygen free radicals in the blood of diabetic patients.

One of the most important features of oils is their thermooxidative stability. Some of the standard techniques that are used to evaluate the stability of mineral or vegetable oils after thermooxidative aging are spectral or colorimetric methods and oxygen absorption. Iftimie et al. [24] have developed a chemiluminescence method for characterizing the thermooxidative stability of oils. A range of mineral and vegetable oils, including olive oil, were subject to the chemiluminescence reaction with luminol as the CL-reagent and a standard method (ASTM-D2272, oxygen absorption in the rotating bomb). During the autooxidative reactions of the hydrocarbon constituents of oils a number of radical species are formed, such as $ROO^{\bullet}$, $RO^{\bullet}$ and $HO^{\bullet}$, similarly to those obtained by the light generating system (luminol and hydrogen peroxide), thus suggesting the possibility of applying the chemiluminescence technique for quantification of oil stability. The oil introduced into the chemiluminescence system shows a quenching effect and the quenching value could be correlated with the stability at the thermooxidative process. The author concluded that chemiluminescence could be used for the determination of the degradation degree.

Reactive oxygen species play a key role in many physiological and pathogenic processes, including signal transduction, inflammation, aging, neurodegeneration and atherosclerosis. At present, intense pharmacological

research is being carried out to find agents that target specific ROS molecules such as H_2O_2. Virgin olive oil is rich in phenolic products, which have been reported to be strong free radical scavengers. Hydroxytyrosol (HT) is a polyphenol extracted from virgin olive oil that has anti-thrombotic activities such as inhibition of LDL oxidation, platelet aggregation and endothelial cell activation. O'Dowd et al. [25] have shown that HT inhibits luminol-amplified chemiluminescence of human neutrophils stimulated with *N*-formyl-methionyl-leucyl-phenylalanine, phorbol myristate acetate and opsonized zymosan. This effect was dose dependent and occurred immediately after the addition of HT. However, HT has no effect on lucigenin-amplified chemiluminescence, suggesting that it does not inhibit NADP oxidase activation or scavenge superoxide anions. The results suggest that HT could exert its antioxidant effect by scavenging hydrogen peroxide but not superoxide anion released during the respiratory burst.

One of the major environmental concerns in the Mediterranean countries is the disposal and/or treatment of the large quantities of olive oil mill wastewater (OMW) produced during olive oil processing. The phenolic compounds inhibit the growth of certain microorganisms, particularly bacteria, and are the major cause, together with fatty acids, of the methanogenic toxicity of OMW. Many authors have observed their phytotoxic effects. Therefore, fast, simple screening of the antioxidant capacity of OMW residues is required, not only to assess their environmental toxicity and the possibility of treating or disposing the waste produced, but also to determine the amount of natural antioxidants present because the olive oil residues can be a cheap source of natural antioxidants. Atanassova et al. [18] have investigated a rapid, simple and sensitive procedure for determining the total phenolic/antioxidant levels of olive oil mill wastewater using Co(II)/ethylenediaminetetracetic acid-induced luminol chemiluminescence. They tested the simple phenolic compound content of olive oil wastewater samples as a function of the extraction system (two- and three-phase centrifugation system). The authors used Co(II) as a catalyst and EDTA as a chelator in order to decrease the speed of the reaction and stabilise the chemiluminescence signal. When the antioxidant activity was measured, adding the antioxidant to the cuvette reduced the stable signal, which served as an index for antioxidant activity. The simplicity of the method lies in the fact that it requires no enzymes and no complicated instrumentation or signal integration procedures, and the chemicals are cheap. The same research group [26] has applied this procedure to OMW samples to evaluate how ultrasonic irradiation can be used to reduce the antioxidant activity of olive oil mill wastewaters. The authors have concluded that low-frequency ultrasonic irradiation can reduce this antioxidant activity.

A study carried out by Buenestado et al. [27] examined the effects of an olive oil-based lipid emulsion [long-chain triacylglycerols-monounsatured fatty acids (LCT-MUFA); ClinOleic] on various functions of human neutrophils in vitro and on rat leukocyte-endothelial cell interactions in vivo compared with LCT (Intralipid) and 50% LCT-50% medium-chain triacylglycerols (MCT; Lipofundin) mixture. Oxidative burst was measured by a chemiluminescence method; cells were suspended in a buffer containing $CaCl_2$, glucose, microperoxidase and luminol. Unspecific quenching of chemiluminescence was excluded by measuring oxidant species production triggered in the absence of cells by xantine oxidase and hypoxanthine. No significant changes of oxidant species production were noticed except for a small transient increase for LCT/MCT. The authors concluded that LCT-MUFA showed lower in vitro and in vivo impact on neutrophil function compared with LCT and LCT-MCT.

TCPO as a CL Reagent

In peroxalate chemiluminescence, the initial excited state product does not emit light at all and instead it reacts with another compound [16]. The chemiluminescence produced by the oxidation of bis(2,4,6-trichlorophenyl)oxlate (TCPO) (Fig. 3) with hydrogen peroxide produces at least one, and possibly two or more, high energy intermediate(s) capable of generating the excited singlet state of fluorescent molecules. The excited fluorophore can lose energy by emitting light and a variety of fluorophors may be used to produce a range of colours.

The peroxide value (PV) is a measure of the total peroxides in olive oil expressed as meq. O_2 kg^{-1} oil, a major guide to quality. A wide variety of analytical methods can be used to determine PV. Stepanyan et al. [28] describes a CL method based on the chemiluminogenic energy-transfer reaction of bis(2,4,6-trichlorophenyl)oxalate (TCPO) with hydrogen peroxide or total peroxides in the presence of Mn(II) as catalyst and 9,10-dimethylanthracene as fluorophore. The PV of model oil samples were measured by the official EU method and the results were favourably

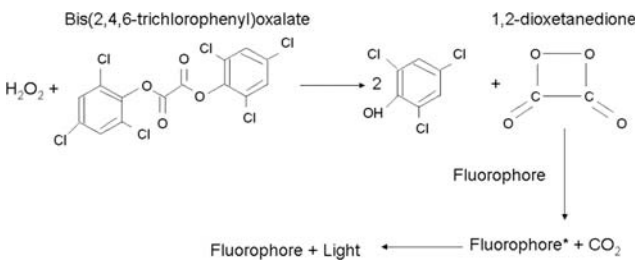

Fig. 3 CL reaction of bis(2,4,6-trichlorophenyl)oxlate

compared with the PO-CL method. The PO-CL method described by the authors is the first CL method that evaluates total peroxides as an analytical index for the quality of olive oil. The instrumentation required is very simple and the measurement can be made manually and outside the laboratory.

Other CL Reactions

Lipid peroxidation in biological membranes is a highly destructive phenomenon that comes about when reactive oxygen species and free radicals attack the double bonds of polyunsaturated fatty acids to yield lipid peroxides. Ruiz Gutierrez et al. [29] examined the effects that the polyphenolic compounds in virgin olive oil—tyrosol, hydroxytyrosol and oleuropein—have on the non-enzymatic lipid peroxidation induced by ascorbate-Fe^{2+} of rat liver microsomes. Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to samples. The inhibition of light emission (maximal induced chemiluminescence) by oleuropein was concentration dependent. Hydroxytyrosol showed a substantial degree of inhibition against ascorbate-Fe^{2+} induced lipid peroxidation in rat liver microsomes that was at least 6 times higher than that observed in the presence of oleuropein. Inhibition of lipid peroxidation by tyrosol was not observed. The results show that oleuropein and hydroxytyrosol are able to prevent lipid peroxidation in rat liver microsomes.

The determination of food authenticity and the detection of adulteration are problems of increasing importance in the food industry. Virgin olive oils are frequently adulterated with other vegetable oils of lower commercial value. Papadopoulos et al. [30] reported a new CL procedure, which can be the basis of more sensitive methods for analysing the potential adulteration or presentation of authenticity factors of olive oils. It can also be used for detecting the adulteration of extra virgin olive oils by seed oils. A weak chemiluminescence emission was observed in commercial extra virgin olive oils and refined seed oils with potassium superoxide in an aprotic solvent. The light was produced by the oxidation of the polyunsaturated fatty acid esters contained in oils (linoleic or linolenic) with superoxide either through excited ketones (direct CL) or after energy transfer to fluorescent compounds (sensitized CL) contained in oils. Oil solutions were prepared in dimethoxyethylene (DME) and the light reactions were started by adding saturated potassium superoxide solution in 1,2-dimethylsulfoxide (DMSO) to the solutions. All the tested edible oils, extra virgin olive oils and sunflower or seed oils were chemiluminescent and the seed oils were 2–3 times stronger than olive oils (linoleic acid concentration in seed oils is several times greater than in virgin olive oils). The considerable differences in CL intensities between seed and extra virgin olive oils are a simple way of determining whether commercial virgin olive oils have been adulterated with cheaper seed oils.

Superoxide anion radicals, one-electron reductants of molecular dioxygen, are the most frequently generated ROS and play a highly significant role in physiology and pathophysiology. The damage caused by ROS can be suppressed by antioxidants. The plant-derived phenolic compounds genistein and oleuropein (the main phenolic compound in the olive) are known to have biological properties which may be the result of their antioxidant and free radical scavenger activity. Kruk et al. [31] report the result of a complex chemiluminescence study of the antioxidant activity of genistein and oleuropein. 18-crown-6 was dissolved in dry DMSO and KO_2 was quickly added to avoid contact with air humidity. Chemiluminescence was used to detect how the tested phenols affected the system generating superoxide anion radicals. When a tested compound was added, the CL signals decreased. This decrease was dependent on the kind of compound added and its concentration. It was highest for oleuropein.

Conclusion

This paper has examined several CL techniques applied to olive oil, which have been of increasing interest in recent years, perhaps because of the good results obtained with CL measurements and the growing interest in the use of olive oil. CL techniques can be used to evaluate antioxidant activity, phenolic levels, radical scavenging and thermooxidative stability. They can also detect possible adulteration by seed oils and determine peroxide values and so act as a guide to quality. Therefore, we can conclude that CL reactions are a reliable procedure for analyzing and monitoring olive oils, and that CL techniques can be used for routine analysis in laboratories

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