Development of a Flow Injection Chemiluminescent Assay for the Quantification of Lipid Hydroperoxides

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ABSTRACT: An automated flow injection chemiluminescence (FICL) system for measuring lipid hydroperoxide (LOOH) concentrations in oils was developed. Initially, a crude oil-in-water emulsion (formed by mixing solvent-diluted oil with the aqueous-based CL compound, luminol, and the catalyst for the reaction, cytochrome c) was tested. The assay was rapid (60 samples per hour), reproducible (CV no greater than 10%, $n = 3$) and had a low sample requirement (1 mg of oil) because of its high sensitivity (0.5 nmol LOOH). CL intensity was influenced by the amount and type of oil under analysis. Owing to these factors, quantitative data were attainable only with a uniform oil concentration and with a calibrant derived from an oil equivalent to that under analysis. This method yielded quantitative data in good agreement with an iodometric titration assay for LOOH ($r = 0.9204$). A refinement of the first method consisted of replacing the luminol and cytochrome c CL compounds with lucigenin, resulting in an assay insensitive to α-tocopherol. A monophasic reaction solution was devised to remove the effect of turbidity; however, the CL signal was still influenced by oil type. Therefore, quantitative data were still attainable only when the same type of oil was used for calibration.

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KEY WORDS: Emulsion, flow injection chemiluminescence, iodometric titration, lipid hydroperoxides, lipid oxidation, lucigenin, luminol and cytochrome c, monophase, peroxide value.

Oxidative rancidity can be considered simply in two stages: the formation of lipid hydroperoxides (LOOH) by, for example, autoxidation, and LOOH decomposition, forming the compounds responsible for the reduction in flavor quality of a food product. The low flavor threshold of the volatiles generated renders even low-fat food items at risk of oxidative rancidity. One approach to monitor the risk of a food item developing oxidative rancidity is to measure the concentration of LOOH. Several methods exist that are based on either the physical properties of the LOOH, e.g., conjugated diene (1), or the chemical properties of the peroxide group, e.g., iodometric titrations (IT) (2). However, limited quantities of oil can impede the analysis of low-fat foods. Chemiluminescence (CL) offers the sensitivity to overcome this problem.

CL is caused by a molecular reaction of two (or more) ground-state molecules producing a final molecule(s) in an excited state. Potential energy from the reactants is translated to the product(s) and, while forming the products, promotes an electron to a higher spin orbital. The energy gained is then lost, *via* photon emission, when the excited-state molecule (product) returns to the ground state. CL can occur with or without the addition of CL reagents, termed indicator-dependent CL and ultra-weak CL, respectively. Several CL methods for detection of LOOH are reported in the literature; however, they provide either rapid qualitative data (3–5) or slower quantitative data (6–8). The use of HPLC combined with postcolumn CL detection has allowed the quantification of various hydroperoxides at the picomole level using a microperoxidase/luminol assay (9,10). Similar reports can be found in the literature for methyl linoleate using a heme/luminol assay (8,11) and a cytochrome c/luminol assay (7). HPLC separation of constituents prior to CL detection adds a level of analytical complexity and places a limitation on the number of samples that can be processed per unit time.

Flow-injection chemiluminescence (FICL) provides CL results by injecting a sample directly into the flow of solvent without prior chromatographic separation. It has many other benefits over static CL and LC-CL approaches (12). It has been used to measure antioxidant activity (13), metal ions (14), pesticides (15), and phenylephrine hydrochloride (16). In all cases, the methods were reported as being sensitive, highly reproducible, and rapid. Despite the rapid reaction kinetics of the CL reaction between LOOH/cytochrome c and luminol, which allows flow cell detection, and the possibility of automation, no one has developed an FICL method to measure the PV of oil. The overall aim of this work was therefore to develop a rapid FICL-based method to measure LOOH in edible oils.

MATERIALS AND METHODS

Materials. The following were obtained from Sigma (Poole, United Kingdom) and used without further purification: ammonium thiocyanate (ACS reagent); borax (sodium tetraborate); chloroform (stabilized with ethanol, 2%); corn oil (CO); cottonseed oil (CSO); cytochrome c (from horse heart, >99%); 1,3-dilinolein (DL; >99%); hydrogen peroxide (aqueous solution, 30%); linoleic acid (LA; >99%); luminol (5-amino-2,3-dihydro-1,4-phthalazinedione); methanol (ACS reagent); peanut oil (PO); soybean oil (SBO); starch (potato); Triton X-100 (*t*-octylphenoxypolyethoxyethanol); *tert*-butyl hydroperoxide (*t*BHPO; aqueous solution, 70%); trilinolein (TL; >99%); and wheat germ oil (WGO). The following were

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obtained from Fisher (Loughborough, United Kingdom) and used without further purification: acetic acid, barium chloride, chloroform (HPLC grade), hydrochloric acid (10.2 N), iron(II) sulfate, iron(III) chloride, methanol (HPLC grade), potassium iodate, potassium iodide, sodium hydroxide (10 N), and sodium thiosulfate.

FICL. The FICL system was adapted from Auerbach and Gray (13), replacing the peristaltic pump with an HPLC pump (PU 980, Jasco) to obtain better reproducibility. An aliquot (50 µL) of sample was injected into a carrier phase of methanol/chloroform (9:1, vol/vol) from a Waters 2690 separation module. The CL reagent consisted of cytochrome c (10 µg mL^{-1}) and luminol (1 µg mL^{-1}) in a borate buffer (25 mM, pH 9.3) containing Triton X-100 (0.5% wt/vol) delivered by an HPLC pump (PU 980, Jasco). The CL reaction proceeded on mixing the sample and the CL reagent, forming a crude emulsion (emulsion-FICL method). An FP920 CL detector (Jasco) was used to measure CL, and the data were recorded by an integrator (Hewlett-Packard 339 2A). The monophasic-based FICL system (monophasic-FICL method) consisted of lucigenin (200 µg mL^{-1}) in a methanol/borate buffer (25 mM) (4:1, vol/vol) solution (pH 10). An aliquot (50 µL) of the WGO samples was injected into the carrier phase (100% methanol). The flow rates of the carrier phase and CL reagent were set at 0.3 mL min^{-1}.

Determination of PV. IT were conducted as reported by Hamilton *et al.* (17). The iron thiocyanate method was conducted as reported by Shantha and Decker (18) using ethanol-stabilized (ES) chloroform as described by Richards and Feng (19). Absorbance readings were conducted using a glass cuvette (1 cm light pathlength). All PV were reported as mmol of LOOH per kg of oil.

Oxidation of oils and lipids. Oil (300 g) was heated (60°C) on a hot plate for 96 h. Samples (20 g) were taken every 24 h and stored at −80°C under an atmosphere of nitrogen in a sealed glass ampule until required. LA, DL, and TL (all 5 g) were heated (80°C) in an incubator oven for 90 min. The lipids were then stored at −80°C under an atmosphere of nitrogen in a sealed glass ampule until required. All samples stored at −80°C were stable to degradation over 1 mon.

Statistical analysis. Experiments were carried out in triplicate and the SD were calculated. ANOVA allowed statistically valid comparisons between data sets to be made. Product–moment correlation coefficients (r) were also calculated when appropriate.

RESULTS AND DISCUSSION

Relationship between t*BHPO concentration and CL intensity.* *t*BHPO was diluted in methanol to give samples with concentrations ranging from 0.1 to 4 µmol mL^{-1}. A linear relationship between *t*BHPO concentration and CL intensity was observed with a high correlation coefficient ($r = 0.9864$). The CV was no greater than 6% ($n = 3$). The data demonstrated that hydroperoxide measurement using the assay was possible, and that the relationship between *t*BHPO concentration and CL intensity was linear over the concentration range studied.

Quantification of LOOH by emulsion-FICL with t*BHPO as calibrant.* The next step was to determine whether the emulsion-FICL method would provide quantitative data, in agreement with the IT method. The IT and the emulsion-FICL methods were used to determine the PV of WGO oxidized over a 96-h period. Emulsion-FICL samples were prepared by dissolving the WGO samples in chloroform (100 mg mL^{-1}). Calibration of the emulsion-FICL method was achieved with *t*BHPO.

The IT method showed an increase in PV with increasing time. At time 0, the PV of the WGO was 0.29 ± 0.20 mmol kg^{-1}. After 24 h of heating, an increase in PV was observed, rising to 18.82 ± 1.69 mmol kg^{-1}. Further heating resulted in a linear increase in PV, reaching a maximum of 150.53 ± 2.10 mmol kg^{-1} after 96 h. Emulsion-FICL analysis of the WGO samples gave a low PV at time 0 h, $0.03 \pm 8.7 \times 10^{-4}$ mmol kg^{-1}, followed by a small increase after 24 h to a PV of 0.09 $\pm 2.12 \times 10^{-3}$ mmol kg^{-1}. A further increase was noted after 48 h of heating, after which a linear increase was observed, reaching a maximum PV of 1.91 ± 0.02 mmol kg^{-1}. Despite the discrepancy in absolute values, both methods showed similar trends in WGO oxidation, having a good correlation coefficient ($r = 0.9204$). Emulsion-FICL analysis showed better reproducibility (CV not greater than 6%, $n = 3$) than the IT method (CV not greater than 9%, $n = 3$).

Differences in the solvents used to dilute the oil samples (chloroform) and *t*BHPO (methanol) calibrants may have been responsible for the significantly lower CL response to oxidized oil than *t*BHPO. The difference in solvent type, and therefore difference in solvent ratio in the detection cell, may have influenced CL intensity. Indeed, solvent type does influence CL intensity. However, the nonpolar solvents are the ones associated with greater CL intensity (20); hence, the differences in solvent type did not explain this result. A more likely explanation is that not all of the LOOH in the WGO samples was detected, as LOOH would need to partition at the oil–water interface of the oil droplets to react with the aqueous-based CL reagents. *t*BHPO is more hydrophilic and would be fully dispersed in the aqueous phase, and thus available to react with the aqueous-based CL reagents. A more hydrophobic calibrant was needed.

Selection of a LOOH calibrant for the emulsion-FICL system. The relationship between LOOH concentration and CL intensity had to be established. Initially, WGO (9 mmol kg^{-1}, as determined by IT) was diluted in chloroform to give samples with concentrations of LOOH ranging from 0.18–3.6 µmol mL^{-1}, and, consequently, with a corresponding range of oil concentrations. The samples were analyzed using the emulsion-FICL method. A nonlinear relationship between the CL response and the LOOH concentration was observed. The emulsion-FICL method had shown a linear relationship between *t*BHPO and CL intensity over a similar concentration range, so the observed trend could not be explained by detection limits. The oil droplet size was not affected by oil

concentration (data not shown). Therefore, we concluded that LOOH exposure to the aqueous-based CL reagents would be equal for all samples, suggesting that another factor was responsible for the nonlinear relationship between LOOH (oil) concentration and CL intensity.

The differences in turbidity between samples might offer an explanation for the nonlinear relationship between LOOH concentration and CL intensity. To observe a linear relationship between LOOH concentration and CL intensity, it was postulated that uniform turbidity between samples was required, i.e., samples with the same oil concentration. Solutions of WGO in chloroform (1 g 50 mL^{-1}) were prepared for both the fresh and heated WGO (PV 1.35 and 169 mmol kg^{-1}, respectively, determined by IT). Portions of the two WGO solutions were mixed, giving LOOH concentrations ranging from 0.027 to 3.38 μmol mL^{-1}. Samples were analyzed using the emulsion-FICL system. The relationship between LOOH concentration and CL intensity was linear (r = 0.9908), where the maximum CV = 6.3% (n = 3). A linear relationship was also observed with PO (r = 0.9511), CSO (r = 0.9905), SBO (r = 0.9861), and CO (r = 0.9856).

The PV of fresh CO, PO, CSO, SBO, and WGO were determined using the IT method. Solutions of each oil type were prepared in chloroform (20 mg mL^{-1}) and analyzed by the emulsion-FICL method. The CL intensities associated with CO, CSO, SBO, and WGO were different (Fig. 1A); only CO and WGO were not significantly different ($P < 0.05$). PO had a CL intensity four times greater than that associated with WGO. A similar effect was reported with fish oils (4). Sodium hypochlorite-induced CL was five times greater with capelin oil than cod liver oil, even though they had the same PV (4). The same study also noted that chromatographically purified fish oil gave CL approximately double that of cod liver oil. In both cases, it was assumed that antioxidants were responsible for the observed effect.

From the above work, we deduced that calibration of the emulsion-FICL method would require an oil equivalent to that under analysis. A calibration curve for the emulsion-FICL method was therefore prepared using both oxidized and fresh WGO diluted with chloroform, to give samples with LOOH concentrations between 0.027 and 3.38 μmol mL^{-1}, with a uniform oil concentration (20 mg mL^{-1}). An increase in the PV of the heat-stressed WGO over time was detected by both methods (Fig. 2A). The CV for the emulsion-FICL method was 5% (n = 3), lower than the CV for the IT method (10%, n = 3). Although differences between the two methods were observed, the correlation coefficient was high (r = 0.9420). The good correlation between the two methods would suggest that quantitative data could be obtained using the emulsion-FICL method, but only under certain conditions.

Refinement of the FICL system. To develop a more versatile FICL method for quantification of LOOH in oil samples, refinements were required to eradicate the problems associated with sample exposure to reagents, phase behavior, and possible interference from compounds in the oil. It has been reported that lucigenin is capable of reacting with hydrogen

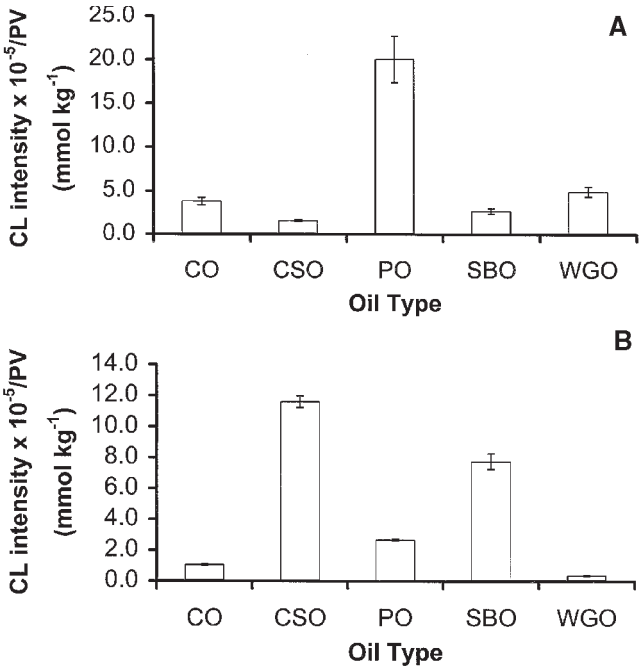

FIG. 1. (A) The effect of oil type on chemiluminescence (CL) intensity measured using the emulsion-flow injection chemiluminescence (FICL) method. The relationship was investigated for corn oil (CO), peanut oil (PO), cottonseed oil (CSO), soybean oil (SBO), and wheat germ oil (WGO). All data are the average of three replicates, where error bars represent SD. Note: All CL intensities are normalized to the PV of the relevant oil as determined by the iodometric titration (IT) method. (B) Effect of oil type on CL intensity measured using the monophasic-FICL method. CO, PO, CSO, SBO, and WGO were diluted in chloroform (20 mg mL^{-1}) and an aliquot (50 μL) of each sample was analyzed using the monophasic-FICL method. All data are the average of three replicates, where error bars represent SD. Note: All CL intensities are normalized to the PV of the oil type, as determined by the IT method.

peroxide without the generation of free radical intermediates (21,22). This may avoid interference from chain-blocking antioxidants such as α-tocopherol (a natural component in oil that may explain some of the oil-dependent CL response observed earlier). Luminol and cytochrome c were replaced by lucigenin, as the latter (unlike the former) was unaffected by α-tocopherol (23). It was also hypothesized that manipulation of the solvent regime from an emulsion to a single (mono-) phase would maximize the exposure of LOOH molecules to the CL reagent, and so promote a linear relationship between LOOH concentration and CL intensity, regardless of oil concentration.

Initial investigations (23) established the ratio of reagents and solvents that gave a monophase within the detector cell, and the effect of solvent type and ratio on CL detector stability. The final system selected was composed of methanol (carrier phase), into which an aliquot of oil diluted in ES chloroform (50 μL) was injected. Lucigenin (100 μmol l^{-1}) was prepared in a methanol/borate buffer (25 mM, pH 10; 4:1, vol/vol). On mixing the sample with the CL reagent, a monophasic solution was formed, with the ratios of the chloroform/methanol/lucigenin solution being 50:40:10 (by vol). On further investigation, we found that the solution remained

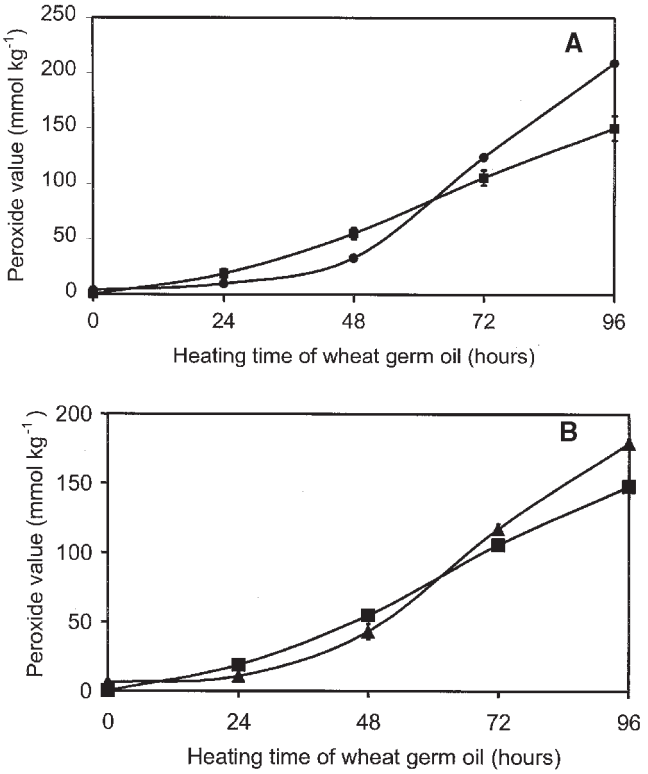

FIG. 2. (A) Comparison of PV measurement by the IT (■) and the emulsion-FICL (●) methods. Calibration of the emulsion-FICL method was achieved using WGO. All WGO samples were prepared in chloroform (20 mg mL^{-1}), and an aliquot (50 µL) analyzed. All points are the average of three replicates, where error bars represent SD (n = 3). (B) Comparison of PV measurement by the IT (■) and monophasic-FICL (▲) methods. Both curves are the average of three replicates, where error bars represent SD. For abbreviations see Figure 1.

monophasic only when oil concentrations below 1% (wt/vol) were used.

The effect of LOOH concentration (varied by altering the amount of oil added to the system) on the CL signal was tested using the monophasic-FICL system. A linear relationship was seen between the LOOH concentration and CL intensity (Fig. 3). The correlation coefficient was high (r = 0.9905) and the CV was no greater than 10% (n = 3). Linear relationships were also discovered with CO, CSO, PO, and SBO (CV values of between 8 and 13%).

Selection of a calibrant for the monophasic-FICL system. The monophasic-FICL method was insensitive to α-tocopherol, and the monophasic solvent regime appeared to allow efficient exposure of LOOH molecules to the CL reagent regardless of the oil type. However, the CL intensity relative to the actual PV was still oil dependent (Fig. 1B). Furthermore, the trend observed differed from that seen with the emulsion-FICL method (Fig. 1A). The reaction kinetics may therefore differ with lipid type. This hypothesis was investigated by comparing the CL associated with oxidized LA, DL, and TL. We assumed that replacing the emulsion system with the monophasic solvent regime in the detection cell would allow equal exposure of LOOH molecules regardless of their chemistry.

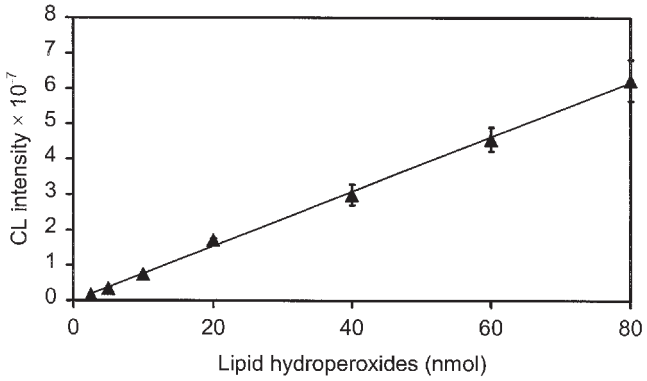

FIG. 3. Effect of lipid hydroperoxide (LOOH) concentration (varied by altering the amount of oil added to the system) on the CL signal in the monophasic-(FICL) system. All points are the average of three replicates, where error bars represent SD. For other abbreviation see Figure 1.

The oxidized lipids were diluted in ES chloroform (20 mg mL^{-1}) and analyzed by the monophasic-FICL method. As noted with the emulsion-FICL system (23), the CL intensity was dependent on the type of lipid (LA > DL > TL) (Fig. 4). However, the difference in CL intensity between the three types of lipid was not as great as that seen with the emulsion-FICL method. Given that the monophasic system allows equal exposure of hydroperoxides to the CL reagent, and given that the high purity (99%) of the lipids minimized any interference from other constituents, different rate constants must exist for different LOOH. The CL intensity associated with PC was 130% greater than with the same concentration of PE hydroperoxide (6), and it was reported that the intensity of CL is dependent on the structure of the hydroperoxide (10). Such problems can be removed through the use of predetection HPLC. This step would remove interfering compounds from the CL reaction and separate LOOH molecules

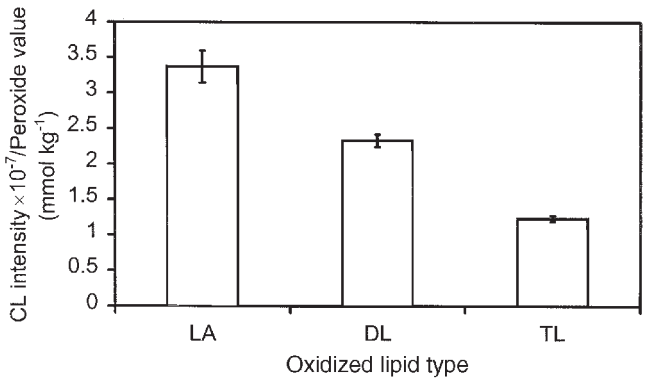

FIG. 4. The effect of LOOH chemistry on CL intensity in the monophasic-FICL. Oxidized linoleic acid (LA), 1,3-dilinolein (DL), and trilinolein (TL) were diluted in ethanol-stabilized chloroform (20 mg mL^{-1}) and an aliquot (50 µL) of each sample was analyzed by the monophasic-FICL method. All CL intensities are the average of three replicates, where error bars represent SD. *Note:* The CL intensities are normalized to the PV of the lipid type as determined by the iron thiocyanate method. For other abbreviations see Figures 1 and 3.

(7). However, the purpose of the work was to develop a rapid method for LOOH quantification, and the introduction of an HPLC step would have increased the run time (*ca.* 15 min).

Despite the advantages of the monophasic system, calibration still requires the use of an oil equivalent to that being studied. A calibration curve for the monophasic-FICL method was prepared using WGO diluted in ES chloroform to give solutions with LOOH concentrations between 0.1 and 4.8 μmol mL^{-1}, again using an aliquot (50 μL) for analysis. Analysis of the heated WGO samples by both the IT and the monophasic-FICL methods (Fig. 2B) showed an overall increase in LOOH concentration with increasing heating time. The two methods did show slight differences in the trend and concentration of LOOH but were similar to those observed in the emulsion system (Fig. 2A). The CV associated with the methods was no greater than 10%, and the correlation coefficient between the two methods was good ($r = 0.9669$).

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