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Development of a novel dual-enzyme screen-printed amperometric biosensor for the analysis of esterified fatty acids

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ABSTRACT

This paper describes, for the first time, the development of a novel trilinolein biosensor based on the immobilisation of lipase and lipoxygenase (LOX) onto a cobalt phthalocyanine screen-printed carbon electrode (CoPC-SPCE) in conjunction with amperometry in stirred solution. The combination of enzymes, integrated into the novel biosensor, provide a selective response for polyunsaturated fatty acid (PUFA) triglycerides, in the presence of both monounsaturated (MUFA) and saturated fatty acid (SFA) triglycerides. The linear range of the trilinolein biosensor is 0.2–10 μM, and the detection limit is 45.5 nM. The biosensor was successfully applied to the measurement of trilinolein present in a pharmaceutical food supplement. The percentage recovery was 86% and the coefficient of variation of the biosensor response was 5.05%. This novel LOX-lipase biosensor is simple to manufacture for trilinolein analysis and has advantages of low cost, speed of analysis and ease of operation, thus making it commercially attractive for a range of applications including food and clinical samples.

1. Introduction

Fatty acids, which predominantly occur as triglycerides, play an important role in food quality, food processing, and have an impact on human health. Linoleic acid (from trilinolein) is of particular interest, as it is one of two essential PUFAs which cannot be synthesised by the body and therefore must be ingested. In humans, linoleic acid and its derivatives show a direct/indirect link with inflammation and metabolic diseases (Choque et al., 2014). The concentration of linoleic acid has implications for the quality and nutritional status of that sample, which is of interest to food producers and consumers (Smart et al., 2020a). It is also of importance in environmental monitoring as a marker of wastewater contamination from the food processing industry by accidental spills of oils. If not treated, they provoke an environmental problem similar to that of petroleum oil due to common physical properties (Čipinytė et al., 2009).

There is a need to develop effective technologies for the detection and quantification of PUFA triglycerides. Currently, these may be analysed by traditional gas chromatographic methods (Seppänen-Laakso et al., 2002); however, these are expensive, time consuming and must be performed in a lab by skilled personnel. In contrast, the novel biosensor approach based on screen-printed carbon electrodes (SPCEs) has many benefits (Smart et al., 2020b). They can be manufactured in a wide-range of geometries at low cost as carbon is an inexpensive material, therefore they can be considered as disposable; and these characteristics lead to rapid, portable and user-friendly devices. Electrocatalysts may be incorporated into the carbon ink of sensors where they act as electron shuttles for electrochemical reactions, thus increase the sensitivity and selectivity of the device (Crew et al., 2004). Furthermore, selectivity is enhanced by incorporating a suitable enzyme onto the surface of the electrochemical transducer. Lipase enzymes are known to hydrolyse triglycerides into free fatty acids (Pohanka, 2019), which therefore makes it a suitable component for inclusion into a novel electrochemical fatty acid biosensor. For the subsequent measurement of the free polyunsaturated fatty acid, the enzyme lipoxygenase (LOX) may also be incorporated into a biosensor to catalyse the oxidation of free PUFAs. The resultant oxidation product, is a fatty acid hydroperoxide. In a previous report (Wring et al., 1992), we first described the electrocatalytic behaviour of several hydroperoxides, namely cumene hydroperoxide and tertiary butyl hydroperoxide, which occurred with a CoPC-SPCE. We believe that this was the first report to show that it was feasible to monitor compounds that contain a hydroperoxide functional group, using a CoPC-SPCE. This research led us to consider exploiting these electrocatalytic reactions, with biosensors, for fatty acid monitoring (Smart et al., 2020a). It should be emphasised that the incorporation of cobalt phthalocyanine into the screen-printed carbon electrode

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(CoPC-SPCE), allows electrochemical measurements to be made at much lower potentials than with unmodified carbon electrodes; this results in improved selectivity of the devices for the target analyte. It should also be mentioned that, in the above paper by Wring et al., 1992, we first reported on the electrocatalytic behaviour of hydrogen peroxide using a CoPC-SPCE. This behaviour has subsequently been exploited as a platform for a variety of electrochemical biosensors, for biomolecules of interest in the agri-food area, where the biorecognition elements are oxidase enzymes (Smart et al., 2020b).

Previous work by our group has demonstrated the feasibility of using screen-printed electrodes in suitable formats as sensors and biosensors for the analysis of target analytes in challenging matrices; for example, for agri-food applications (progesterone in milk (Pemberton et al., 2001), monosodium glutamate in stock cubes (Hughes et al., 2014), fructose in fruit juice (Nicholas et al., 2018), organophosphates in cereals and raw produce (Crew et al., 2004), boar taint in pork (Westmacott et al., 2020), and thiamine in soft drinks (Smart et al., 2019). Other research groups have also exploited advantages of electrochemical sensors for the analysis of biomolecules in challenging matrices (Wu et al., 2021; Li et al., 2021; Xia et al., 2023; Li et al., 2022a; Li et al., 2022b; Li et al., 2022c; Li et al. 2020).

It should be mentioned that SPCE-based biosensors have been used successfully for commercial applications, including glucose monitors, which have revolutionised diabetes care and have been commercially successful (Wang, 2001). SPCE biosensors have also been recognised by other groups as a powerful analytical tool due to their versatility, other applications include organophosphate pesticide detection (Renedo et al., 2007; Pundir and Chauhan, 2012; Pohanka et al., 2012), metals in water (Alvarado-Gámez et al., 2015; Sanllorente-Méndez et al., 2010); (Domínguez-Renedo et al., 2009), antibiotics in milk (Talib et al., 2018) and yeast in wine (Borisova et al., 2018). As in most glucose biosensors, the use of mediators, also known as electrocatalysts, has greatly improved the selectivity by allowing a lower operating potential to be used.

The aim of this investigation was to explore the possibility of developing a robust, electrochemical, biosensor for the esterified form of a polyunsaturated fatty acid (linoleic acid), known as trilinolein. It was of particular interest to develop a suitable strategy for the immobilisation of two enzymes, namely lipase, and lipoxygenase, on to the surface of a screen-printed carbon electrode, impregnated with the electrocatalyst cobalt phthalocyanine (CoPC-SPCE); several methods of enzyme immobilisation were investigated in the construction of the biosensor. A CoPC-SPCE was incorporated into the biosensor as it produces an electrocatalytic response with the end product of the enzyme reactions, namely linoleic hydroperoxide, the electrocatalytic response enhances the selectivity of the biosensor. The application of the proposed biosensor to the successful determination of trilinolein in a food supplement is described.

This paper will discuss the fabrication and application of a novel, screen printed biosensor for the measurement of trilinolein in a food supplement, in order to deduce the suitability of the biosensor in the agri-food sector. The development of the electrochemical biosensors for triglyceride analysis is the subject of a UK patent application (Hart et al., 2022).

2. Materials and methods

2.1. Chemicals and reagents

Conjugated linoleic acid (CLA) capsules were purchased from Holland and Barrett (Nuneaton, UK); five capsules were opened and their contents mixed. All other chemicals were purchased from Sigma Aldrich (Dorset, UK). Deionised water was obtained from a Purite RO200 Stillplus HP System (Oxon, UK). Stock solutions of monosodium, disodium and trisodium orthophosphate were prepared at a concentration of 0.2 M by dissolving the appropriate mass in deionised water; these were then titrated to achieve the desired pH and diluted in the cell to achieve a working concentration of 0.1 M. Sodium chloride was prepared to a concentration of 1.0 M by dissolving the appropriate mass in deionised water; this was diluted in the cell, giving a final concentration of 0.1 M.

Aliquots of LOX and lipase solutions were diluted with 0.1 M pH7 phosphate buffer saline to give the desired number of enzyme units. A 50% glutaraldehyde stock solution was diluted with 0.1 M pH7 phosphate buffer saline give a 0.01% solution. Stock solutions of linoleic acid, trilinolein and CLA from capsules were prepared by dissolving the required mass in ethanol to achieve 1 mM solutions.

2.2. Instrumentation

All voltammetric and amperometric measurements were carried out with a μAutolab III potentiostat interfaced to a PC for data acquisition via NOVA v2.0 (Metrohm, Barendrecht, The Netherlands) or an AMEL Model 466 polarographic analyser attached to an ABB Gorez SE120 chart recorder. An in-house low pass filter (time constant 22 s) was incorporated between the potentiostat and the chart recorder to substantially reduce stirrer noise.

CoPC-SPCEs are commercially available and were supplied by Gwent Electronic Materials Ltd. (Pontypool, UK). The working electrode was fabricated using a carbon-based ink with CoPC (C2030408P3) and the reference electrode was fabricated using a Ag/AgCl ink (C2130809D5). The working electrode area (3 mm × 3 mm) was defined using electrical insulation tape.

All pH measurements were performed using a Testo 205 (Testo Limited, Alton, Hampshire UK) pH meter. Solutions were stirred using a colour squid (IKA, Tunbridge Wells, UK) and warmed using a HAAKE P5 water bath (Thermo Scientific, Loughborough, UK).

Surface morphology and composition of the working electrode were analysed using a Quanta FEG 650 scanning electron microscope (FEI, Hillsboro, OR, USA) (4000 × magnification; samples were gold-coated). Aztec energy dispersive X-ray microanalysis (EDX) (Oxford Instruments, Abingdon, UK) was then performed on these samples at 20 kV, using × 300 magnification and 689.9 × 455.9 μM area. Further characterisation of the biosensor surface by Fourier transform infra-red spectrometry using a Spectrum Two FT-IR Spectrometer in conjunction with a diamond/ZnSe crystal attenuated total reflectance attachment (Perkin Elmer, Waltham, USA).

2.3. Biosensor fabrication and storage

For the fabrication of the LOX-lipase biosensors, the surface of the CoPC-SPCE working electrodes were coated with the enzymes in buffer solution using three different strategies; a) 10 μL containing 15 U of LOX followed by 10 μL containing 45 U of lipase, b) 10 μL containing 45 U of lipase followed by 10 μL containing 15 U of LOX, or c) 10 μL containing 15 U of LOX and 45 U of lipase. Each enzyme layer was dried overnight using a vacuum desiccator at 4 °C prior to any subsequent additions. Therefore, all the final biosensors contained 15 U of LOX and 45 U of lipase.

The dried enzyme layers were subsequently cross-linked to the electrode surface by coating the enzyme layer with 10 μL of 0.01% glutaraldehyde solution onto the enzyme layers and dried overnight in a vacuum desiccator at 4 °C. All biosensors were stored in airtight containers at 4 °C for up to 3 months to evaluate the storage stability of the biosensors.

2.4. Amperometric and voltammetric procedures

In order to deduce the optimum operating potential for amperometric measurements in stirred solution using the optimally fabricated LOX-lipase biosensor, a hydrodynamic voltammogram was constructed over the range + 0.0 to + 1.2 V vs. Ag/AgCl using 100 μM of linoleic acid

(from 33.3 μM of trilinolein) in 10 mL 0.1 M pH8 phosphate buffer saline. The solution was stirred at 250 rpm at 37 °C.

A calibration study was performed with the LOX-lipase biosensor in conjunction with amperometry in stirred solution at + 0.5 V vs. Ag/AgCl. Ten 20 μL additions of 1 mM trilinolein were made into a cell containing 10 mL pH 8 0.1 M phosphate buffer saline, stirred at 250 rpm at 37 °C. A low concentration calibration study was performed using an analogue instrument with a low pass filter to reduce stirrer noise. Ten 2 μL additions of 1 mM trilinolein were added into a cell containing 10 mL 0.1 M pH 8 phosphate buffer saline solution, stirred at 250 rpm at 37 °C. A new biosensor was used for each calibration study.

The method of standard addition was used in conjunction with the LOX-lipase biosensor to determine the percentage recovery of trilinolein that was present in the CLA capsules. The biosensor was inserted into the cell containing 10 mL 0.1 M pH8 phosphate buffer saline. Amperometry in stirred solution was performed at + 0.5 V vs. Ag/AgCl, and the cell was stirred at 250 rpm at 37 °C. A solution containing trilinolein solution from CLA capsules was added to the cell at a theoretical concentration of 3 μM; this was followed by five identical additions from the 1 mM linoleic acid standard, each representing 2 μM additions to the cell concentration. The steady-state currents were measured after each addition and the data used to construct a standard addition plot from which the unknown concentrations were deduced. This procedure was repeated for five separate aliquots from individual CLA capsules, each with a new biosensor, and the resulting data used to calculate the recovery and precision.

The selectivity of the biosensor was ascertained by examining a selection of potentially interfering endogenous species found in food. A cell containing 10 mL 0.1 M pH8 phosphate buffer saline, stirred at 250 rpm and warmed to 37 °C was prepared. A CoPC-SPCE was used in conjunction with amperometry in stirred solution at + 0.5 V vs Ag/AgCl. Additions of α-linolenic actable id, thiamine, pyridoxine, niacin, pantothenic acid, riboflavin, folic acid, cobalamin, biotin, malic acid, citric acid and ascorbic acid were sequentially added to the cell at regular intervals; in each case, three 10 μL additions of the 10 mM stock solution of each reagent was made to the cell. This was repeated with α-linolenic acid, retinol, ergocalciferol, cholecalciferol, α-tocopherol, vitamin K1 and vitamin K2, and again with α-linolenic acid, glycine, taurine alanine, cysteine, tryptophan and tyrosine. At the end of each study, all potential interferents were present in the cell with α-linolenic acid.

The effect of storage on LOX-lipase biosensor performance was assessed by performing calibration studies using biosensors stored for different lengths of time. Five 20 μL additions of 1 mM trilinolein were made into 10 mL 0.1 M pH8 phosphate buffer saline; amperometry in stirred solution was used in conjunction with a CoPC-SPCE containing 45 U of lipase and 15 U of LOX, at 0.5 V vs. Ag/AgCl, 37 °C and 250 rpm.

It should be noted that the biosensors have been designed for single-use and therefore individual biosensors were replaced following each measurement.

3. Results and discussion

3.1. Operation, fabrication and evaluation of the LOX-lipase biosensor

Fig. 1 shows the sequence of reactions involved in the operation of the dual-enzyme amperometric biosensor. When trilinolein is added to the cell containing the biosensor, the first enzyme reaction, involving lipase, converts the triglyceride to the corresponding free fatty acid linoleic acid (FFA). This latter species then undergoes a second enzyme reaction, involving LOX, to produce linoleic hydroperoxide (ROOH). This hydroperoxide then diffuses to the screen-printed CoPC-SPCE surface where it undergoes an electrocatalytic reaction involving cobalt phthalocyanine. During this process, the Co^{2+} centre of the organometallic species is first chemically reduced to Co^{+} by ROOH, which is then electrochemically oxidised back to Co^{2+} at the electrode surface, which is held at a potential of + 0.5 V vs Ag/AgCl. This latter electron transfer

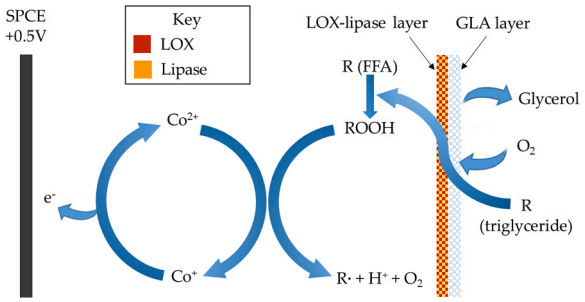

Fig. 1. Reaction scheme for the measurement of trilinolein using a lipase-LOX containing CoPC-SPCE biosensor. The cations Co+ and Co2 + are the redox forms of the organometallic electrocatalyst cobalt phthalocyanine which is integrated within the SPCE. Other abbreviations are: glutaraldehyde (GLA); free fatty acid (FFA).

reaction constitutes the response current.

For the fabrication of the biosensor (Fig. 2), lipase and LOX were deposited onto a base CoPC-SPCE transducer in three different fabrication methods prior to the glutaraldehyde cross-linking step: 1) an initial lipase layer was deposited followed by a LOX layer; 2) a LOX layer was deposited followed by a lipase layer; 3) a single combined LOX-lipase layer by drop-coating a mixed solution onto the electrode. All three biosensors contained 15 U of LOX and 45 U of lipase. The LOX enzyme loading was previously optimised, as reported in (Smart et al., 2020); whilst the lipase loading was calculated to be in excess.

It was observed that the biosensors produced by depositing the enzymes separately resulted in poor adherence to the sensor surface, resulting in flaking of the deposited material, whereas the combined enzyme layer proved to be strongly adhered to the working electrode. The performance of the proposed biosensor was evaluated using calibration studies over the range of 2–10 μM of trilinolein; amperometry in stirred solution at + 0.5 V vs Ag/AgCl showed that the triple-layer biosensors had poor precision; a coefficient of variation (CoV) of 22.32% for lipase followed by LOX followed by glutaraldehyde, and a CoV of 19.07% for LOX followed by lipase followed by GLA), whereas the mixed enzyme layer biosensors had the best precision (CoV of 5.44%) and produced the most reproducible and sensitive response (Table 1). This data strongly supports our observations that the mixed-enzyme deposition resulted in the most structurally stable immobilised enzymatic layer. On further examination, the multi-layer enzyme deposition strategies resulted in layers with poor adherence to the working electrode. Therefore, further studies used biosensors prepared

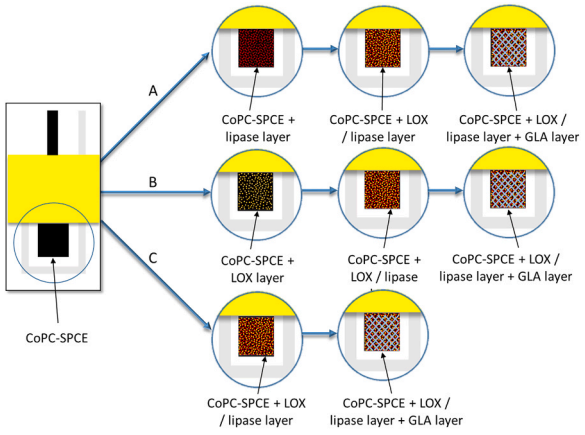

Fig. 2. Schematic diagram of the three strategies for layer-by-layer fabrication of the dual enzyme biosensor prior to applying the glutaraldehyde cross-linking layer; A) an initial lipase layer followed by a LOX layer; B) a LOX layer followed by a lipase layer; C) a single combined LOX-lipase layer.

Table 1
Reproducibility and sensitivity of different fabrication methods for a LOX-lipase CoPC-SPCE biosensor, for the measurement of 10 μM trilinolein using amperometry in stirred solution.

Fabrication method	Coefficient of variation (%, n = 3)	Sensitivity
LOX layer followed by lipase layer	19.07	y = 4.593x
Lipase layer followed by LOX layer	22.32	y = 5.732x
Mixed enzyme layer (LOX and lipase)	5.44	y = 5.778x

All biosensors contained 15 units of LOX and 45 units of lipase, and contained a final layer of glutaraldehyde.

by the deposition of the mixture comprising LOX and lipase onto the CoPC-SPCE. A linear relationship was observed between concentration of trilinolein and current response, demonstrating that the biosensor can be used to directly measure trilinolein in solution, avoiding the need to add lipase to the solution.

Scanning electron microscopy (SEM) was used to investigate the surface morphology of the selected LOX-lipase biosensor. The morphology was similar to that previously seen for a LOX-only biosensor fabricated in a similar manner (Smart et al., 2020). A cohesive outer film was observed, which is attributed to the cross-linking agent glutaraldehyde (Fig. 3). The porous nature of the glutaraldehyde allows ingress of the analyte but retains the enzymes within the reaction layer; this is indicated by the steady state responses (see later section on Performance Characteristics and Fig. 6). The surface morphology of the base CoPC-SPCE transducer used in the present study has previously been thoroughly characterised using SEM and energy dispersive X-ray spectroscopy (Smart et al., 2019). The SEM images showed characteristic flakes of carbon, it was clear that the CoPC-SPCE had a porous three dimensional surface structure. The elemental composition showed the main component present was carbon (88%), cobalt was also present (0.3%) from cobalt phthalacyanine which was added to the ink before screen-printing (Table 2).

Further characterisation of the surface of the proposed biosensor, produced by crosslinking lipase-LOX with glutaraldehyde, was performed using FTIR spectroscopy. The FTIR spectrum obtained for glutaraldehyde itself (Fig. 4), clearly shows a strong, sharp band at 1725 cm^{-1} which results from the C═O stretching mode. However, the spectrum obtained for the biosensor surface (Fig. 4) does not exhibit this band, which indicates that reaction of the C═O with the enzymes amino group has occurred; this is supported by the appearance of a band in the spectrum of the biosensor at 1650 cm^{-1}, indicating the presence of a C═N group. Therefore, the FTIR study indicates that cross linking of glutaraldehyde with the enzymes has occurred resulting in the formation of a schiff base. Similar observations, and conclusions, have been

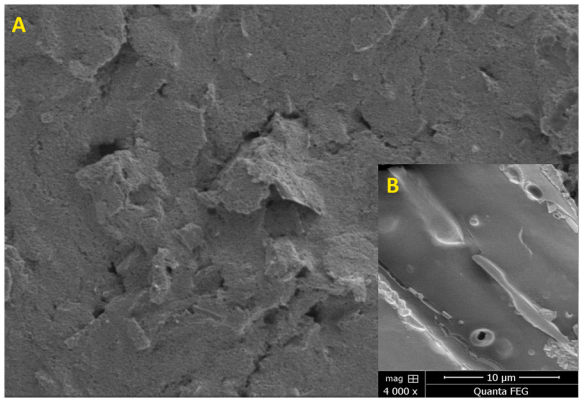

Fig. 3. SEM images at 4000x magnification; A) the surface of the CoPC-SPCE electrode; B) the CoPC-SPCE a single combined LOX-lipase layer cross-linked to the electrode surface with glutaraldehyde.

Table 2
Energy dispersive X-ray microanalysis (EDX) of CoPC-SPCEs.

Element (% by weight)	Mean (standard deviation) n = 3
Carbon	88.195 (0.379)
Oxygen	3.029 (0.101)
Sulphur	0.189 (0.017)
Chlorine	8.209 (0.342)
Cobalt	0.317 (0.014)
Bromine	0.060 (0.008)

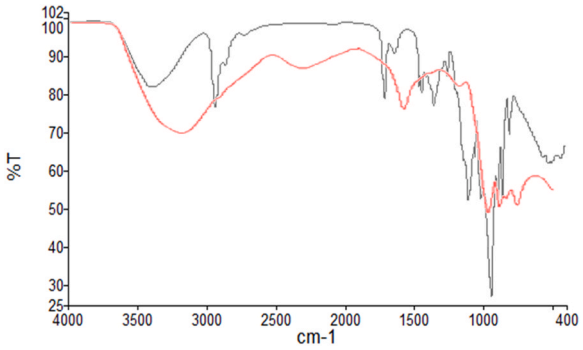

Fig. 4. FTIR analysis of the surface of the CoPC-SPCE electrode with glutaraldehyde only (blue line) and the CoPC-SPCE with a single combined LOX-lipase layer cross-linked to the electrode surface with glutaraldehyde (pink line).

reported previously, in an FTIR study, where the enzyme glucose oxidase was cross linked with glutaraldehyde for immobilisation onto a sensor surface (Lović et al., 2018).

Hydrodynamic voltammetry was performed with the LOX-lipase CoPC-SPCE biosensor with a final concentration of linoleic acid of 33.3 μM of trilinolein producing 100 μM of free linoleic acid (Fig. 5). An electrocatalytic oxidation response, arising from the hydroperoxide species, occurs from + 0.3 V to + 0.7 V (see Fig. 1 for reaction sequence). This is followed by a further oxidation response beginning at + 0.75 V, which continues to increase in magnitude to at least 0.95 V. We believe this latter response is a result of the direct oxidation of the hydroperoxide species at the plain SPCE surface (Smart et al., 2020). Consequently, the operating potential of + 0.5 V vs. Ag/AgCl was selected for further work in this investigation; this value would provide better selectivity than higher potentials whilst producing good sensitivity (Table 3).

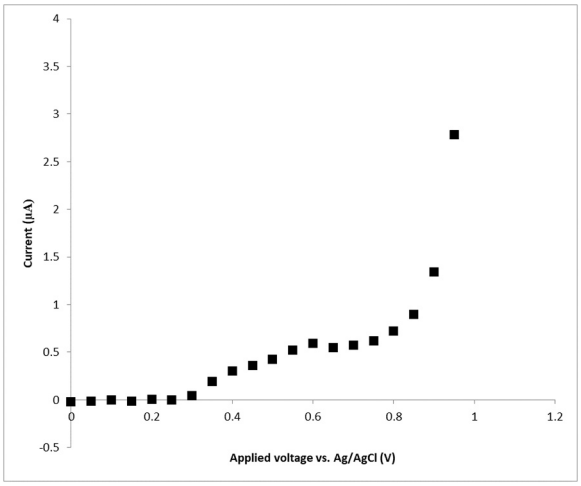

Fig. 5. Hydrodynamic voltammogram of a CoPC-SPCE biosensor containing 15 units of LOX and 45 units of lipase, in a 33.3 μM solution of trilinolein.

Table 3
Responses of potential interfering naturally occurring species obtained using the LOX-lipase CoPC-SPCE biosensor in conjunction with amperometry in stirred solution at the optimised measurement conditions for trilinolein.

Class	Potential Interferent	Response (as a % of α-linolenic acid response)
Water soluble vitamins	Vitamin C (ascorbic acid)	Yes (665%)
	Vitamin B1 (thiamine)	No
	Vitamin B2 (riboflavin)	No
	Vitamin B3 (niacin)	No
	Vitamin B5 (pantothenic acid)	No
	Vitamin B6 (pyridoxine)	No
	Vitamin B7 (biotin)	No
	Vitamin B9 (folate)	No
	Vitamin B12 (cobalamin)	No
Fat soluble vitamins	Vitamin A (retinol)	Yes (47%)
	Vitamin D2 (ergocalciferol)	No
	Vitamin D3 (cholecalciferol)	No
	Vitamin E (α-tocopherol)	No
	Vitamin K1	No
	Vitamin K2	No
Amino acids	Tryptophan	Yes (326%)
	Glycine	No
	Taurine	No
	Alanine	No
	Cysteine	No
	Tyrosine	Yes (263%)
Acid regulators	Malic acid	No
	Citric acid	No

3.2. Performance characteristics of the LOX-lipase biosensor

Initially, a calibration study was performed to deduce the linear range of the triglyceride biosensor with trilinolein. An analogue instrument, in conjunction with a low-pass filter, was used to eliminate stirrer noise, and allow the measurement of reliable steady state measurements in the μM concentration range. Amperometry in stirred solution was performed at + 0.5 V vs Ag/AgCl, and the resulting calibration plot is shown in Fig. 6; clearly a linear response was obtained over a concentration range of 0.2–10 μM of trilinolein (the equation for the calibration plot is y = 5.4033x - 0.8129). The limit of detection can be deduced from measuring 3 times the noise of the raw amperometric data (Fig. 6), and this was calculated at 45.5 nM. Intra-sensor reproducibility was calculated by measuring the steps from the amperogram in Fig. 6, and the CoV was 8.9%. These performance characteristics compare very favourably with other lipase-containing biosensors which have previously been developed for triglyceride determination (Table 4).

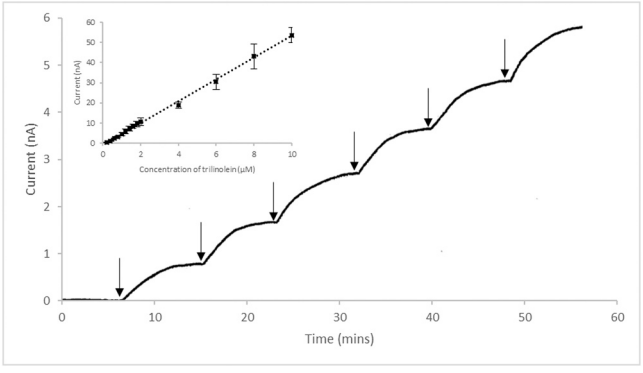

Fig. 6. Amperogram showing individual additions of trilinolein, indicated by arrows, using a CoPC-SPCE biosensor with 45 units of lipase and 45 units of LOX, in conjunction with amperometry in stirred solution at + 0.5 V vs. Ag/AgCl. The top left insert shows the generated calibration plot (R^2 =0.998).

The selectivity of the above biosensor was examined using amperometry in stirred solution with a variety of endogenous occurring species, likely to be present in food (Table 3).

Of the 23 species that were examined (Table 3), only four gave a measurable response at the concentration studied, however this was much higher than would normally be expected in real samples. Therefore, the selectivity of this device is considered to be suitable for measurements in complex matrices.

A review by Pohanka (2019) contains reports of lipase-based electrochemical biosensors for triolein measurement; the fabrication and performance characteristics of these biosensors are summarised in Table 4.

Previous biosensors based on platinum (Narang, 2008), pencil graphite (Narwal and Pundir, 2017) and glassy carbon (Madhurantakam et al., 2020) are unsuitable for commercialisation, and furthermore have multiple fabrication steps containing three or more enzymes. The approach described in the current paper is simpler, requiring fewer fabrication steps and only two enzymes in a single deposition layer followed by a crosslinking agent.

The novel biosensor is also advantageous in its selectivity for PUFA triglycerides, as only PUFAs interact with LOX to form hydroperoxides, which are detected electrocatalytically. Pijanowska et al. (2001) reported a pH-electrode-based biosensor, which measures pH change when free fatty acids are liberated from triglycerides by lipase. However, this pH change will occur for all classes of fatty acid triglycerides. The biosensors described by Narang et al., 2009, Narwal and Pundir (2017) and Madhurantakam et al. (2020) are based on detection of hydrogen peroxide generated from glycerol; glycerol will be generated from all classes of fatty acid triglycerides by the action of lipase. Therefore, none of the reported biosensors are selective for PUFA triglycerides.

The LOX-lipase biosensor, developed by our group, was evaluated in this study using commercial food supplement, CLA capsules, as an example agri-food sample. A standard addition method was used in conjunction with amperometry in stirred solution using an operating potential of + 0.5 V vs Ag/AgCl (Table 5); five separate replicate analyses of the capsule were carried out, using five separate biosensors. The percentage recovery (Table 5, column 4) was very good, averaging 86% with a co-efficient of variation of 5.05%. The improved performance characteristics are due to simpler preparation steps and operation of the analytical methods. The simplicity of fabrication and reproducibility of the measurements make the proposed biosensor attractive for commercial analytical applications.

3.3. Storage study

The performance of the lox-lipase biosensor over time was assessed by performing calibration studies of trilinolein at monthly intervals over a period of 3 months of storage in a refrigerator (4 °C) following fabrication (Fig. S1). At 3 months of storage, there was no decrease in sensitivity; the slopes at each time point were not statistically significantly different from each other (t-test; $p > 0.05$). This suggests the potential for an extended shelf-life. The linear range was also the same over each time point (2–10 μM). This suggests this technology holds promise for mass production and commercialisation.

4. Conclusion

In the present study, a novel LOX-lipase biosensor was fabricated using a simple drop-coating procedure, whereby a mixture consisting of lipase and LOX was deposited onto the surface of a CoPC-SPCE. A convenient simple immobilisation method using glutaraldehyde maintained the stability of the biosensor without loss of enzyme or enzyme activity. The LOX-lipase biosensor showed favourable performance characteristics for trilinolein measurement, with a wide linear range of 0.2–10 μM, and a low limit of detection of 45.5 nM. This biosensor also

Table 4
Performance characteristics of lipase containing biosensors for triglyceride analysis.

Biosensor fabrication	Analyte	Limit of Detection	Linear range	Potential for Commercialisation?	Ref
Lipase and lipoxygenase on a screen-printed carbon electrode	Trilinolein	45.5 nM	0.2–10 μM	Yes	Our approach
Lipase on an ion selective field effect transistor pH electrode	Triacetin, tributyrin and triolein	Around 1 mmol/L	Up to 30 mM	No	Pijanowska et al., 2001
Lipase, glycerol kinase, glycerol-3-phosphate oxidase and horseradish oxidase on Pt electrode	Triolein	0.11 mM	0.56–2.25 mM	No	Narang et al., 2008
Lipase, glycerol-kinase and glycerol-3-phosphate oxidase nanoparticles on pencil graphite electrode	Triolein	0.1 nM*	0.1–45 mM	No	Narwal and Pundir (2017)
Lipase, glycerol kinase, glycerol-3-phosphate oxidase and adenosine triphosphate on glassy carbon electrode with carbon nanotubes and graphene	Triolein	0.019 mM	0.5–5.5 mM	No	Madhurantakam et al., 2020 [26]

*This is a theoretical value calculated from the slope of a calibration plot using mM standards (which are 10^6 higher than the quoted LoD)

Table 5
Percentage recovery of trilinolein in a pharmaceutical food supplement using a LOX-lipase (bio)sensor.

N	Theoretical conc.*	Measured conc.	% recovery
1	3.0 μM	2.6 μM	86.7
2	3.0 μM	2.5 μM	83.3
3	3.0 μM	2.5 μM	83.3
4	3.0 μM	2.8 μM	93.3
5	3.0 μM	2.5 μM	83.3
Mean			86.0
sd			4.35
CoV			5.05

*Theoretical concentration of trilinolein from contents of capsules which manufacturers claim contains 80% conjugated linoleic acid

has the advantage over other technologies as it is selective for PUFA fatty acid triglycerides, due to the combination of LOX and lipase enzymes. It was demonstrated that trilinolein could be successfully determined in a pharmaceutical food supplement, as an example agri-food sample; the average recovery was 86.0% with a corresponding coefficient of variation of 5.05%. No pre-treatment of the sample is required, which greatly reduces the analysis time compared with other methods for triglyceride analysis. Furthermore, the use of the biosensor is the more convenient method and therefore suitable for use by non-technical personnel. It is readily feasible that the new trilinolein biosensor may be applied to a range of food types, and has potential for clinical analysis. The storage stability data and high reproducibility make these devices attractive for commercialisation.

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Declaration of Competing Interest

The authors do not have any conflict of interest to declare.

Data Availability

The data presented in this paper submission also forms part of a patent application with the reference: Hart, J. P., Crew, A., Smart, A., Doran, O. (2022). 'Biosensor for Triglycerides'. Patent Application GB2202278.4 (Applicant: University of the West of England). United Kingdom: IPO. Consequently, the data will unfortunately not be available until the patent application is published in the public domain, but will be available on publication of the patent application/patent.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2023.105488.

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