



Ergometrine sensing in rye flour by a magnetic bead-based immunoassay followed by flow injection analysis with amperometric detection

Soraya Höfs^{a,b,*}, Valerie Jaut^a, Rudolf J. Schneider^c

^a Department of Analytical Chemistry; Reference Materials; Bundesanstalt für Materialforschung und -prüfung (BAM); Richard-Willstätter-Straße 11, 12489, Berlin, Germany

^b Institute for Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Straße 24 – 25, 14476, Potsdam, Germany

^c Technische Universität Berlin, Straße des 17. Juni 135, 10623, Berlin, Germany

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ABSTRACT

A certain group of mycotoxins, the ergot alkaloids, has caused countless deaths throughout human history. They are found in rye and other cereals and ingesting contaminated foods can cause serious health problems. To identify contaminated food exceeding the legal limits for ergot alkaloids, a portable and cost-effective test system is of great interest to the food industry. Rapid analysis can be achieved by screening for a marker compound, for which we chose ergometrine. We developed a magnetic bead-based immunoassay for ergometrine with amperometric detection in a flow injection system using a handheld potentiostat and a smartphone. With this assay a limit of detection of 3 nM ($1 \mu\text{g L}^{-1}$) was achieved. In spiked rye flour, ergometrine levels from 25 to 250 $\mu\text{g kg}^{-1}$ could be quantified. All results could be verified by optical detection. The developed assay offers great promise to meet the demand for on-site ergometrine detection in the food industry.

1. Introduction

Since time immemorial, humans and animals are exposed to mycotoxins mainly through ingestion of fungus-infested foods and plants. Throughout human history, a group of mycotoxins, the ergot alkaloids, have repeatedly caused epidemic-like outbreaks, resulting in tens of thousands of deaths in Europe in the Middle Ages [1]. Ergot alkaloids are produced by parasitic fungi of the *Claviceps* genus [2], most commonly by *Claviceps purpurea* [2,3], but also other fungus species such as *Aspergillus* and *Penicillium* are known to produce them [2]. Infection of plants such as grasses and grains leads to replacement of grains by the wintering body of the fungi, called sclerotia, which is dark, hardened mycelium containing the ergot alkaloids [4]. The highest levels of ergot alkaloids are usually found in rye and rye products [3,5], but also other cereals as wheat, barley, triticale, millet and oat can get infected by *Claviceps purpurea* [3].

Today, more than 50 different ergot alkaloids are known [3] and the content in sclerotia is highly variable [3,6–8]. Ergometrine, ergosine, ergocornine, ergotamine, ergocristine, ergocryptine (α - and β -isomers) and their corresponding -inine (S)-epimers are of particular importance due to their high abundance in sclerotia [3]. Young and Chen reported a

relative composition of ergot alkaloids in Canadian rye, wheat, triticale, and barley of ergocristine 31% – ergocristinine 13%, ergotamine 17% – ergotaminine 8%, α -ergo-cryptine 5% – α -ergocryptinine 3%, ergometrine 5% – ergometrinine 2%, ergosine 4% – ergosinine 2%, ergocornine 4% – ergocorninine 2% [9].

The ergoline moiety is the common structural feature of the major ergot alkaloids, methylated at the N6 nitrogen, with a C9–C10 double bond, which is variably substituted at the C8 position (see Fig. 1) [2]. In contrast to ergometrine/-inine, which is a simple lysergic acid derivative, ergosine, ergocornine, ergotamine, ergocristine, ergocryptine and their -inine forms are peptide alkaloids (ergopeptines) [10].

Ergot poisoning, called ergotism or St. Anthony's fire is characterized by vasoconstriction and consequent gangrene causing the loss of fingers, hands, feet, or even entire limbs [11]. Other symptoms include abdominal pain, nausea and vomiting, insomnia or hallucinations [12]. Today fatal ergot alkaloid poisoning is rare, since ergot sclerotia can be effectively removed by color sorting machines. The presence of ergot sclerotia is a good indicator of the presence of ergot alkaloids. However, the absence of ergot sclerotia does not necessarily guarantee the absence of ergot alkaloids [5]. A reason is that broken sclerotia can contaminate other grains [13]. Therefore, in 2021, a new regulation of the European

* Corresponding author. Department of Analytical Chemistry; Reference Materials; Bundesanstalt für Materialforschung und -prüfung (BAM); Richard-Willstätter-Straße 11, 12489, Berlin, Germany.

E-mail addresses: soraya.hoefs@bam.de (S. Höfs), rudolf.schneider@bam.de (R.J. Schneider).

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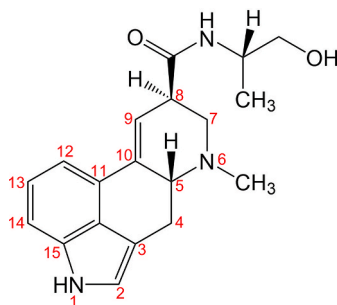


Fig. 1. Structure of ergometrine.

Commission on the maximum levels of ergot alkaloids in foodstuffs came into force, regulating not only the maximum levels of ergot sclerotia but also the content of the sum of the six major ergot alkaloids and their corresponding epimers [13]. For instance, a legal limit of $500 \mu\text{g kg}^{-1}$ applies to rye milling products until June 30, 2024, which will be lowered to $250 \mu\text{g kg}^{-1}$ from July 1, 2024 on [13]. Consequently, the development of analytical procedures allowing to quantify ergot alkaloids, is of particular importance.

High-performance liquid chromatography with fluorescence (HPLC-FLD) or tandem mass spectrometry (HPLC-MS/MS) detection provide reliable methods for sensitive detection of the six major ergot alkaloids and their corresponding epimers [3]. Many of the reported chromatographic methods enable to quantify the major ergot alkaloids individually [14–17], allowing to sum up the individual values to obtain the total ergot alkaloid content. However, this requires many costly calibration standards. A more cost-effective approach is to transfer the ergot alkaloids into a uniform structure before quantitative analysis is performed. A promising method for obtaining a common structure, reported by Kuner et al., is to cleave the ergot alkaloids by hydrazinolysis [18]. Here, ergotamine, ergocristine, ergosine, ergocornine, α -ergocryptine and their corresponding epimers could be cleaved to lysergic acid hydrazide [18].

Beside chromatographic methods, which are predominantly important for official and reference laboratories to guarantee consumer safety, there is a high demand for cost-effective rapid test systems that can be applied at the production site to perform a first screening for ergot alkaloids. Consequently, contaminated samples can be removed from the production before any further time-consuming and costly analysis is performed. Enzyme-linked immunosorbent assays (ELISA) provide a solid basis for sensitive and rapid screening for ergot alkaloids. Significant work on competitive ergot alkaloid ELISAs has been performed in the 1990s by Shelby et al. [19–22]. Competitive ELISAs for ergotamine or ergometrine were developed based on different antibodies produced with an ergotamine and ergometrine protein conjugate as immunogen, respectively [19,20]. For the specificity of the antibodies used in immunoassays, the position of the conjugation on the ergot alkaloid molecule to a protein used for the immunization is of particular importance [22,23]. Arsens and Zenk demonstrated with lysergic acid derivatives that the conjugation of a protein via the imino group of the indole results in highly specific polyclonal rabbit antibodies against lysergic acid, whereas the conjugation via the carboxylic group produces rather generic antibodies that can bind also other simple lysergic acid derivatives, such as ergometrine [23]. Nevertheless, in radioimmunoassays, the obtained generic antibodies had only low cross-reactivities with ergopeptines [23]. Also, some other studies could demonstrate, that antibodies directed against the ergoline moiety are much more specific to lysergic acid or ergometrine than to ergopeptines [20,24,25].

To the best of our knowledge, all the reported ergot alkaloid immunoassays relied on traditional optical read-out in microtiter plate format or radioactivity measurements and no sensors or rapid test for ergot alkaloids have been reported so far. In contrast, numerous sensing approaches for all other mycotoxins regulated by the European

Commission (aflatoxins [26,27], ochratoxin A [28,29], patulin [30], deoxynivalenol [27,31], zearalenone [27,32], fumonisins [27,33], T-2 [27] and HT-2 toxin [34]) have been described in literature, giving great promise for future on-site analysis [35,36]. For this purpose various types of sensors were applied, such as lateral flow tests [27], amperometric lab-on-a-chip biosensors [26], impedimetric [28] or conductometric sensors [30] in which mostly antibodies [26,27,36] but also aptamers [28,36], molecular imprinted polymers (MIPs) [36] or enzymes [30] were used as recognition elements.

In this work we want to demonstrate an approach for ergot alkaloid detection which is closer to application in practice. As reported by us earlier, magnetic bead-based immunoassays provide a fast alternative to classical ELISAs [29,37], and electrochemical methods are well-suited to meet the demand for on-site detection systems. Recently we have developed a straightforward approach to implement portable electrochemical detection for immunoassays using horseradish peroxidase (HRP) as signaling enzyme and 3,3',5,5'-tetramethylbenzidine (TMB) + H_2O_2 as substrates [29]. The required enzyme conjugate was based on poly-HRP-streptavidin and lysergic acid to correspond with the commercially available anti-ergometrine antibody. Yet, for this magnetic bead-based immunoassay, we conceived a novel tracer consisting of a lysergic acid mimic, a polyethylene glycol (PEG) spacer with 8 units, and a terminating biotin (PEG(8)-biotin) to enable for the detection of one of the ergot alkaloids, ergometrine, in rye flour.

2. Material and methods

2.1. Materials

Tween™ 20 and 3,3',5,5'-tetramethylbenzidine were purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). Magnetic Dynabeads™ Protein G for Immunoprecipitation (Invitrogen) with a diameter of $2.8 \mu\text{m}$ and Poly-HRP Streptavidin were obtained from Thermo Fisher Scientific). Screen-printed gold electrodes (250AT) were obtained from Metrohm DropSens (Oviedo, Asturias, Spain). Sodium citrate monobasic, BioXtra, anhydrous, $\geq 99.5\%$, sodium chloride, BioUltra, $\geq 99.5\%$, potassium phosphate dibasic anhydrous, BioUltra, $\geq 99.0\%$, potassium phosphate monobasic, $\geq 99.0\%$, sodium phosphate dibasic dihydrate, $\geq 99.0\%$, sodium dihydrogen phosphate dihydrate, $\geq 99\%$, potassium sorbate, purum p.a., $\geq 99.0\%$, tris(hydroxymethyl)aminomethane (TRIS), ACS reagent, *N,N*-dimethylacetamide (DMA), 99.8%, tetrabutylammonium borohydride (TBABH), 98%, sulfuric acid, ACS reagent, 95.0–98.0%, acetonitrile anhydrous, 99.8%, ergocristine, phyproof® reference substance, and hydrogen peroxide solution, $\geq 30\%$, for trace analysis, were obtained from Merck KGaA (Darmstadt, Germany). Potassium hexacyanoferrate(III), 98+% ($\text{K}_3[\text{Fe}(\text{CN})_6]$), and potassium chloride, ACS, 99.0–100.5%, were bought from Alfa Aesar (Tewksbury, MA, USA). Albumin fraction V, biotin-free (NZ-Origin), was purchased from Carl Roth (Karlsruhe, Germany). Ergometrine and ergotamine were purchased from LGC Standards (Teddington, Middlesex, UK). Lysergic acid–poly(ethylene glycol)(8)–biotin (lysergic acid–PEG(8)–biotin) was synthesized by ASCA GmbH (Berlin, Germany). Mouse monoclonal IgG1 anti-ergot alkaloid antibodies (Anti-Ergot Alkaloides Antibody, 1 mg mL^{-1}) were purchased from BioTeZ Berlin-Buch GmbH (Berlin, Germany). Polyclonal sheep anti-mouse IgG antibodies IgG (R1256P) were purchased from Acris Antibody GmbH (Herford, Germany). For ELISA, transparent high-binding 96-well microplates (polystyrene) from Greiner Bio-One (Solingen, Germany) were used and for the magnetic bead-based immunoassay transparent non-binding 96-well microplates (polystyrene) from Corning (Corning, NY, USA) were applied. Ultrapure water from a Milli-Q water purification system (Merck Millipore) was used. Organic rye flour (type 997) was obtained from a local supermarket in Berlin.

2.2. Flow injection system

A 30 mL luer lock syringe (Omnifix® Solo, B. Braun, Melsungen, Germany) was connected via Teflon tubing to a manual six-port injection valve (RH 7725i IDEX, Oak Harbor, WA, USA). A syringe pump (model Fusion 720, Chemyx, Stafford, TX, USA) was used to adjust the flow rate of the buffer (between 0.5 and 4 mL min⁻¹). A 300- μ L sample loop was built from Teflon® tubing and connected to the injection valve. To remove air bubbles from the flowing solution, a bubble trap (Darwin Microfluidics, Paris, France) was connected upstream to the wall-jet flow cell. Comprehensive information on the wall-jet flow cell was reported by us earlier [29]. For amperometric measurements, the sample loop was overfilled with at least 600 μ L of each sample to ensure complete filling. Thereafter, the sample was manually injected into the running flow of buffer by flipping the switch of the injection valve.

2.3. Amperometric measurements

For amperometric measurements a handheld potentiostat EmStat3 Blue (PalmSens, Houten, The Netherlands) was used and connected to a laptop or an android smartphone. The flow cell was electrically shielded by a faraday cage (Metrohm, Herisau, Switzerland). All electrochemical experiments were performed in the flow injection system described above and with screen-printed electrodes (Metrohm DropSens, Oviedo (Asturias), Spain) with a gold working electrode, platinum counter electrode and silver pseudo reference electrode. To each sample and each buffer, KCl was added to obtain a 100 mM concentration of chloride ions stabilizing the potential of the reference electrode. The influence of the flow rate on the current signal was studied by injecting 10 μ M potassium ferricyanide into the running flow of 100 mM potassium phosphate buffer (pH 7 with 100 mM KCl). To avoid any error, the potassium ferricyanide samples were prepared in the same buffer. For each flow rate at least three injections were performed.

2.4. Ergometrine ELISA

The competitive ELISA for ergometrine was carried out in transparent high-binding 96-well microtiter plates. Each incubation step was performed at room temperature (RT) while shaking at 750 rpm on a Titramax 101 plate shaker (Heidolph, Schwabach, Germany). Washing of the plates was performed with a plate washer (ELx405 Select™, BioTek Instruments, Bad Friedrichshall, Germany) using wash buffer (0.75 mM potassium dihydrogen phosphate, 6.25 mM dipotassium hydrogen phosphate, 0.025 mM sorbic acid potassium salt, 0.05% (v/v) Tween™ 20, pH 7.6) and three cycles per washing step. A 40 mM TMB stock solution, containing 8 mM TBABH was prepared under argon in DMA and stored in the dark at 4 °C. Ergometrine, ergotamine and ergocristine calibration standards were prepared from 0.5 mg mL⁻¹ stock solutions. The ergometrine stock solution was prepared under argon in acetonitrile: water, 90:10 (v/v) and the ergotamine and ergocristine stock solutions were prepared in acetonitrile under argon. Aliquots of each stock solution were stored at -20 °C in the dark.

First, the plates were coated with secondary anti-mouse antibody from sheep. 200 μ L of antibody solution (1 mg L⁻¹ in PBS, pH 7.6) were added to each well and incubated for 16 h. After washing, each well was filled with 300 μ L of biotin-free bovine serum albumine (BSA) solution (10 mg mL⁻¹ in PBS, pH 7.6) and incubated for 1 h to block the surface. Subsequently, the plate was washed again and incubated for another hour with 200 μ L per well of primary mouse anti-ergot alkaloid antibody (1.33 ng mL⁻¹, in PBS, pH 7.6). Then, the plate was washed again and incubated for 30 min with ergometrine (or ergotamine, or ergocristine) standards in Tris buffer (10 mM Tris, 150 mM NaCl, pH 8.5) with 150 μ L per well and the lysergic acid PEG(8)-biotin tracer (7.5 ng mL⁻¹ in Tris buffer: 10 mM Tris, 150 mM NaCl, pH 8.5) with 50 μ L per well. The tracer was added immediately after the standards or after 5 min, 10 min or 15 min of substrate incubation to test the influence of pre-incubation

with the analyte on the sensitivity of the assay. The plate was washed again and poly-HRP-streptavidin (20 ng mL⁻¹, in PBS, pH 7.6) was added, using 200 μ L per well. After 30 min the plate was washed a final time and incubated for 20 min with 200 μ L of TMB/H₂O₂ in citrate buffer (360 μ M TMB, 3.7 mM H₂O₂ in 220 mM sodium citrate buffer, pH 4). Finally, 100 μ L of 1 M H₂SO₄ were added to each well to stop the reaction and the OD was measured with a microplate reader (SpectraMax® i3, Molecular Devices, San José, CA, USA) at a wavelength of 450 nm referenced to 620 nm. The arithmetic mean values of the measured ODs were plotted against the concentration and a four-parameter logistic function was fitted to the data. (The optimized tracer and antibody concentrations were obtained by diluting both components as far as possible while still achieving a sufficient OD value around 1. Therefore, different tracer and antibody concentrations were tested in the ELISA described above, without the addition of ergometrine standards, see Fig. S2.)

2.5. Magnetic bead-based immunoassay for ergometrine

For the magnetic bead-based immunoassay for ergometrine, transparent non-binding 96-well microtiter plates were used. To bind the anti-ergot alkaloid antibodies, protein G decorated magnetic beads (2.8 μ m) were used. Each incubation step was performed under shaking at RT and 1000 rpm on a Titramax 101 plate shaker (Heidolph, Schwabach, Germany). To wash the beads, wash buffer (as described above) was added to the beads manually with a multi-channel pipette and removed under magnetic capturing of the beads. This was repeated twice for each washing step. To capture the beads, a plate separator (BioMag® 96-well plate separator, Polysciences, Warrington, PA, USA) was applied.

For one assay (in a 96 well plate), 240 μ L protein G decorated magnetic beads were used (30 mg mL⁻¹). The particles were captured with a magnet in a 2 mL reaction tube, and the supernatant of the bead dispersion was removed. Subsequently, 6 μ L of anti-ergot alkaloid antibody solution (1 mg mL⁻¹) and 1.2 mL PBS (pH 7.6) were added to the reaction tube and incubated with the beads for 30 min under shaking at 1000 rpm and 23 °C with a ThermoMixer (Eppendorf, Germany). Subsequently, the beads were magnetically captured to remove the solution and washed for three times with 500 μ L of wash buffer. The beads were then dispersed in 33 mL PBS (pH 7.6) by vortexing and 300 μ L of the bead dispersion were added to each well of a microplate. The beads were captured with the magnetic plate separator to remove the supernatant. Immediately afterwards, the ergometrine standard solutions (in 10 mM Tris, 150 mM NaCl, pH 8.5) were added to the beads with 150 μ L per well. After 15 min of pre-incubation, 50 μ L of the lysergic acid PEG (8)-biotin tracer (7.5 ng mL⁻¹ in Tris buffer, 10 mM Tris, 150 mM NaCl, pH 8.5) were added to each well, containing the beads and ergometrine standards, and incubated for another 15 min. Next, the beads were washed as described above and 200 μ L of poly-HRP streptavidin (20 ng mL⁻¹, in PBS, pH 7.6) were added to each well and incubated for 30 min. The beads were washed again and 200 μ L of TMB/H₂O₂ in citrate buffer (360 μ M TMB, 3.7 mM H₂O₂ in 220 mM sodium citrate buffer, pH 4) were added to each well. After 10 min, 100 μ L of 0.9 M H₂SO₄ with 0.3 M KCl were added to each well to stop the reaction and to adjust the chloride ion concentration of the samples to 100 mM. The OD was measured with a microplate reader (SpectraMax® i3, Molecular Devices, San José, CA, USA) at a wavelength of 450 nm referenced to 620 nm.

Amperometric measurements were performed directly after optical detection with the flow injection system and electrochemical set-up described above (see 2.2 and 2.3). To this end, the samples were filled into a 1 mL syringe (Injekt®-F Luer Solo, B. Braun, Melsungen, Germany). To ensure complete filling of the sample loop of the flow injection system, three replicates were combined to obtain a sample volume of approximately 900 μ L. As a running buffer for amperometric measurements, TMB/H₂O₂ substrate solution (360 μ M TMB, 3.7 mM H₂O₂ in 220 mM sodium citrate buffer, pH 4) was mixed with the stop solution (0.9 M H₂SO₄, 0.3 M KCl) in a 2:1 ratio. The potential was set to 300 mV

vs. Ag/AgCl and a flow rate of 4 mL min⁻¹ was used. The current was measured over time and the first sample was injected as the baseline current reached a steady state. After the baseline current returned to the steady state, further samples were injected.

2.6. Detection of ergometrine in spiked rye flour samples

Organic rye flour samples were spiked with 50 ng, 125 ng, 250 ng, and 500 ng of ergometrine in 200 μ L PBS (pH 7.6) per 2 g sample. After 1 h, 10 mL of PBST (Phosphate-buffered saline with 0.05% Tween, pH 7.6) were added to each sample and carefully vortexed for 2 min. To perform matrix-matched calibration, the extraction with PBST was also performed with unspiked rye flour. Subsequently the samples were centrifuged at 10 000 rpm for 10 min and the supernatants were used for further analysis with the magnetic bead-based immunoassay. The extracts were diluted 1:5 with PBS (pH 7.6). The assay was carried out as described above (see 2.5) but using the diluted extracts of the unspiked flour to prepare the ergometrine standards for the calibration curve and PBS as diluent for the lysergic acid PEG(8)-biotin tracer (7.5 ng mL⁻¹, pH 7.6). To analyze the spiked samples, the diluted sample extract was added to the antibody-decorated beads and the immunoassay was carried out as described above.

3. Results and discussion

3.1. Principle of the magnetic bead-based immunoassay for ergometrine

A competitive direct immunoassay format on magnetic beads was developed to quantify ergometrine (see Fig. 2 a). Monoclonal mouse anti-ergot alkaloid antibodies were bound by simply mixing them with protein G-modified magnetic beads under shaking for 30 min. Protein G has a specific affinity to the Fc region of mouse antibodies [38] and therefore no further coupling reagents are necessary. This offers a quick

alternative to the time-consuming overnight antibody-coating steps of conventional ELISAs in microtiter plates. Unbound antibodies can be removed by washing the beads under magnetic capturing.

A novel tracer for this anti-ergometrine antibody was conceived, consisting of a lysergic acid mimic, a polyethylene glycol (PEG) spacer with 8 units, and a terminating biotin (PEG(8)-biotin). The antibody-coated beads were incubated with the ergometrine-containing sample and lysergic acid PEG(8)-biotin as a tracer. After 30 min, the beads were washed and incubated for another 30 min with poly-HRP labeled streptavidin. Finally, the beads were washed again and incubated with the HRP substrates TMB/H₂O₂. After 10 min, the reaction was stopped by the addition of sulfuric acid and the detection of the fully oxidized yellow-colored TMB can be performed optically by measuring the optical density (OD) statically in a microtiter plate or amperometrically in a flow injection system.

Fig. 2 b gives a schematic overview of ergometrine detection in spiked rye flour samples. For this purpose, ergometrine was extracted with PBST and the diluted extract was analyzed with the magnetic bead-based immunoassay. The final reaction mixtures containing the fully oxidized TMB were analyzed in our custom-made flow injection system with amperometric measurements by a handheld potentiostat connected via Bluetooth to a smartphone.

3.2. Amperometric detection in a flow injection system

Earlier we have reported on the pitfalls of the electrochemical TMB detection at different pHs (pH 1 and pH 4) and working electrode materials (gold and carbon). It was demonstrated, by cyclic voltammetry on screen-printed gold electrodes, that the electrochemistry of TMB has a reversible character at pH 1 undergoing a single oxidation and reduction, whereas it is rather irreversible at pH 4 [29]. Therefore, screen-printed gold electrodes are well suited to perform amperometric detection of fully oxidized TMB at pH 1 and a potential of 300 mV vs.

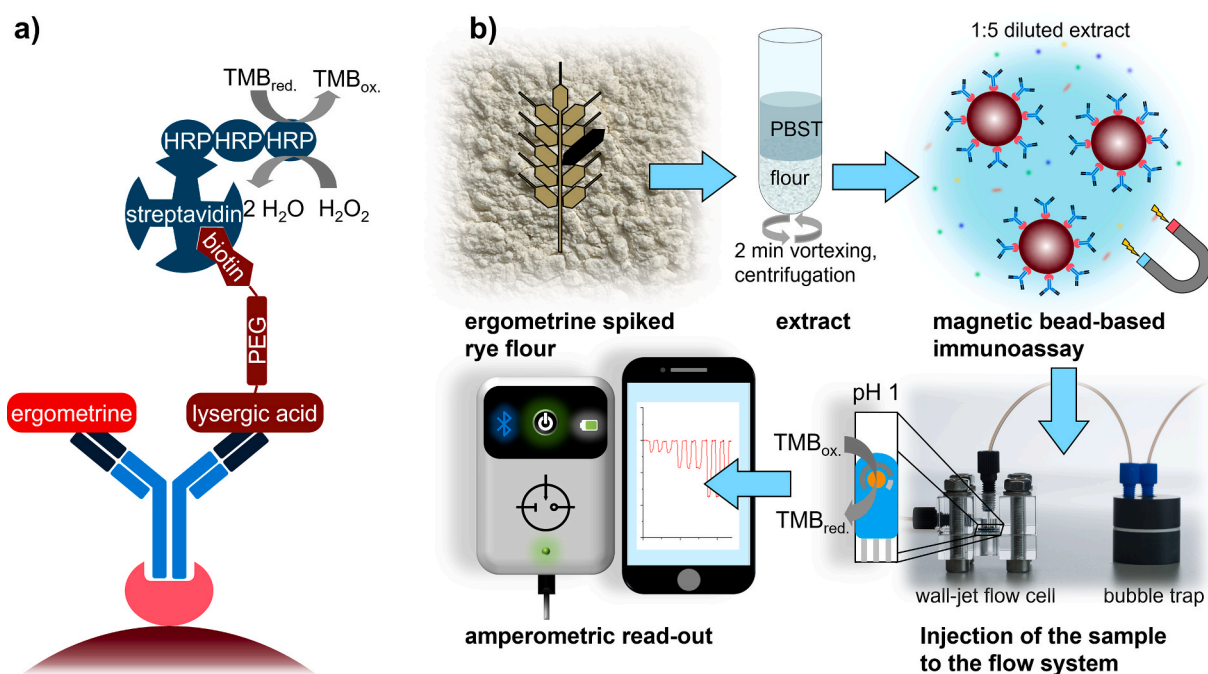


Fig. 2. a) Principle of the magnetic bead-based immunoassay for ergometrine. Protein G decorated beads were used to capture the anti-ergot alkaloid antibodies. Lysergic acid PEG(8)-biotin was used as a tracer, competing with ergometrine for the binding sites at the antibody. For signal generation, poly-HRP-streptavidin was bound to the biotin moiety of the tracer and TMB/H₂O₂ were used as enzymatic substrates. TMB was enzymatically oxidized, and the reaction was stopped by the addition of sulfuric acid generating the fully oxidized TMB (yellow diimine). b) Procedure to determine ergometrine in spiked rye samples. A short extraction with PBST and 2 min vortexing was performed. The samples were centrifuged for 10 min and the 1:5 diluted extract was analyzed by the magnetic bead-based assay as shown in a). The samples containing the fully oxidized TMB are injected into the flow system with a wall-jet flow cell and a screen-printed gold electrode. TMB is reduced at pH 1 and 300 mV vs. Ag/AgCl and the current is measured with a handheld potentiostat connected via Bluetooth to a smartphone.

Ag/AgCl [29]. Here, each sample, alternating with buffer, was pumped through our custom-made wall-jet flow cell until the current reached a steady state. Measuring the steady state current has the advantage of good precision when manually controlling the injected volume, but it is quite time-consuming. Furthermore, this approach requires a relatively high sample volume. In contrast, the use of a flow injection system with a sample loop allows to precisely control the injected volume, and consequently transient current can be used as a signal. This has two advantages: on one hand, the injected sample volume can be reduced, and, on the other hand, samples can be analyzed in shorter time.

Since short analysis time is crucial in food analysis, we have focused on reducing the detection time in amperometric measurements. Therefore, our custom-made wall-jet flow cell for screen-printed electrodes was installed into a flow injection system, depicted in Fig. 3 a. A six-port manual injection valve with a 300 μL sample loop was connected to the flow cell via Teflon tubing. A bubble trap was connected upstream of the flow cell to remove air bubbles from the flowing solution. The flow injection system was operated with a single syringe pump, allowing to set different flow rates.

Typically, in amperometric measurements, a sufficient overpotential is applied to the electrode, that the current is limited by mass transport. As mass transport to the electrode can be improved by forced convection, the flow rate in amperometric measurements influences the current densities. Yamada and Matsuda first reported that the limiting current in wall-jet electrodes is, *inter alia*, proportional to the power of 0.75 of the volume flow rate [39]. Hence, the current density can easily be increased to some extent by increasing the flow rate. Here, the influence of different flow rates ranging from 0.5 to 4 mL min^{-1} on the current signal in amperometric measurements was studied with our flow system using potassium ferricyanide as redox molecule. To this end, ferricyanide was reduced at the electrode at a potential of 0 V vs. Ag/AgCl. For each of the tested flow rates, the same injection volume of 300 μL , defined by the sample loop, was used. It could be demonstrated that the current peak height increases with the flow rate, while the peak width decreases (see Fig. 3 b). Fig. 3 c shows the highly reproducible peak height. Thus, it can be concluded that higher flow rates allow improved mass transport and therefore higher currents. Furthermore, the total measuring time is shorter, without the need of waiting until the current reaches a steady state leading to an increased sample volume. For all following experiments, a flow rate of 4 mL min^{-1} was used. Under the chosen experimental conditions one sample can be analyzed in around 45 s (including the time for the manual injection).

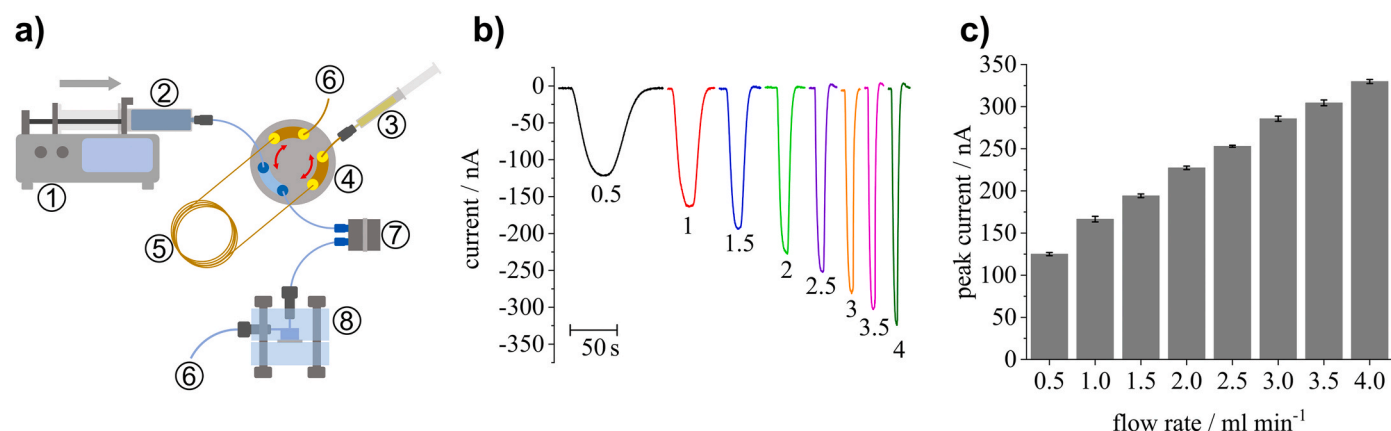


Fig. 3. a) Flow injection system consisting of: (1) syringe pump, (2) syringe filled with buffer, (3) syringe with sample for injection, (4) six-port manual injection valve, (5) sample loop, (6) waste, (7) bubble trap, (8) wall-jet flow cell with screen-printed electrode. b) Amperometric current signals obtained with the flow injection system and screen-printed gold electrodes at 0 V vs. Ag/AgCl. Samples of 300 μL 10 μM potassium ferricyanide in 100 mM potassium phosphate buffer with 100 mM KCl (pH 7) were injected at different flow rates ranging from 0.5 to 4 mL min^{-1} . c) Peak currents obtained for different flow rates in amperometric measurements as depicted in b) ($n = 3$).

3.3. Magnetic bead-based immunoassay with amperometric detection

For comparison, a direct competitive ELISA has been developed using a commercially available monoclonal mouse anti-ergot alkaloid antibody, lysergic acid PEG(8)-biotin as tracer and poly-HRP to generate the signal with TMB and H_2O_2 . After addition of H_2SO_4 optical read-out was performed. With this competitive assay format, a C-value (inflection point of the curve) of $25.5 \pm 0.8 \text{ nM}$ ($8.3 \pm 0.3 \mu\text{g L}^{-1}$) could be achieved (see Fig. S3 a).

The mouse anti-ergot alkaloid antibodies were produced with a lysergic acid-BSA conjugate as the immunogen. Consequently, a high affinity of the antibody to lysergic acid derivatives, such as ergometrine, could be expected. To test whether the antibody can also bind the structurally different ergopeptines, two different ergopeptines, ergotamine and ergocristine, were tested for cross-reactivity in the developed ELISA (see Fig. S3 a). Relatively low cross-reactivities of 7% for ergotamine and <0.1% for ergocristine were determined. Consequently, the tested antibody is well-suited for the development of quite specific ergometrine immunoassays. Low cross-reactivities with ergopeptines were reported also in other studies using antibodies directed against the ergoline moiety (using lysergol [24] or lysergic acid protein conjugates as immunogens [23]).

To improve the sensitivity of the assay, the microplate, coated with primary antibody, was pre-incubated with ergometrine standards before the tracer was added. This favors the binding of the analyte rather than the tracer and can therefore shift the C-value to lower concentrations. Here the antibody-coated plate was pre-incubated with standards for 5, 10, or 15 min before adding the tracer, which was incubated for a further 25, 20 or 15 min, respectively. It was observed that the C-value decreased from $26 \pm 9 \text{ nM}$ ($9 \pm 3 \mu\text{g L}^{-1}$) after immediate addition of the tracer to $12 \pm 3 \text{ nM}$ ($3.8 \pm 0.8 \mu\text{g L}^{-1}$) upon addition after 15 min (see Fig. S3 b). Longer pre-incubation times were not tested, as this would further reduce the incubation time with the tracer which results in the loss of signal intensity. For all further experiments, including the magnetic bead-based immunoassay described below, a pre-incubation time with ergometrine standards or samples of 15 min was used.

The monoclonal mouse anti-ergot alkaloid antibody was further used to develop the magnetic bead-based immunoassay for ergometrine. The flow injection system was used to perform the amperometric detection of oxidized TMB, and the results were compared with optical detection by measuring the OD. For amperometric detection of TMB, optimized reaction conditions were used as reported by us earlier for another HRP-based immunoassay (magnetic bead-based immunoassay for Ochratoxin A) [29]. In short, a potential of 300 mV vs. Ag/AgCl was applied to the

screen-printed gold electrode and the current was measured over time at pH 1 to reduce fully oxidized TMB in the presence of all other reaction substrates (TMB/H₂O₂).

To compare the electrochemical with the optical method, calibration of the magnetic bead-based immunoassay has been performed with different ergometrine concentrations ranging from 0.1 nM to 1.23 µM and the signals were measured photometrically and amperometrically. Fig. 4 shows the amperometric curve obtained for the calibration with 12 samples, detecting the fully oxidized TMB. With this it could be demonstrated that the read-out for 12 samples can be performed in less than 10 min (~45 s per sample).

The calibration curve obtained from three replicates per each ergometrine standard is depicted in Fig. 5 a, and Fig. 5 b shows the corresponding results of the optical detection. C-values of 13.8 ± 0.5 nM (4.5 ± 0.17 µg L⁻¹) and 13.8 ± 0.7 nM (4.5 ± 0.2 µg L⁻¹) were obtained with amperometric and optical detection, respectively. Accordingly, the obtained results are very similar to those achieved with the classical microplate ELISA described above, but the applicability of the magnetic bead-based immunoassay has significant advantages as no overnight incubation and blocking of the surface is required. Moreover, the background signal obtained with the magnetic bead-based immunoassay is much lower than that of the classical microplate ELISA. This can be concluded by comparing both methods with optical detection (see also Fig. S3). In literature, the reported C-values for classical competitive microplate ELISAs or a radioimmunoassay for ergometrine range from 0.5 to 100 nM [20,23,25,40].

For each calibration curve, the relative error of the concentration was calculated and plotted vs. the concentration, which allows to create a precision profile for the calibration range, first described by Ekins [41] (see Fig. 5 a and b). It was fixed here that the measurement range of the assay should be defined as the range within which the relative concentration error should not exceed a limit of 30%. Consequently, ergometrine could be quantified between 3 nM (1 µg L⁻¹) and 300 nM (100 µg L⁻¹) by amperometric read-out and from 6 nM (2 µg L⁻¹) to 1.2 µM (400 µg L⁻¹) by optical read-out. Both detection methods provide high precision in the central part of the calibration curve (i.e., around the inflection point), and the limits of detection (LOD) are approximately the same for both techniques. However, the optical read-out has better precision at high ergometrine concentrations. Due to the inversely proportional relationship of the signal (current or OD) and the ergometrine concentration, signals are lower at high ergometrine concentration. In electrochemical measurements this results in relatively low currents of less than 50 nA which might cause the higher relative error in

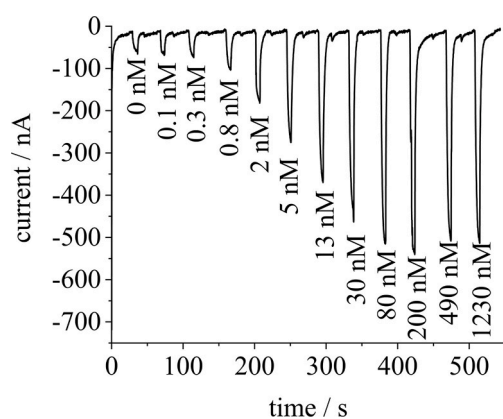


Fig. 4. Amperometric curve for the read-out of the magnetic bead-based immunoassay for different ergometrine concentrations (0–1230 nM) obtained with the flow injection system. The samples containing the fully oxidized TMB are injected sequentially. Redox peaks result from the reduction of TMB at 300 mV vs. Ag/AgCl at screen-printed gold electrodes. All samples and the running buffer contain 150 mM sodium citrate, 300 mM H₂SO₄, 100 mM KCl, 240 µM TMB and 2.5 mM H₂O₂.

comparison to optical detection. However, for the application of the assay, the quantification of low ergometrine concentrations is of far greater importance. Fig. 5 c shows the electrochemical vs. the optical signal. The signals from both methods correlate very well. The results demonstrate that the electrochemical detection of TMB is a reliable alternative to optical read-out in immunoassays.

3.4. Detection of ergometrine in spiked rye flour

The highest levels of ergot alkaloids are usually found in rye and rye products [3,5]. A beneficial application of the magnetic bead-based immunoassay for ergometrine therefore would be to use it in the detection of ergometrine as an indicator compound in rye flour, since no reference materials are available in this context, spiked rye flour was analyzed. To this end, rye flour was spiked with different concentrations of ergometrine (25, 62.5, 125 and 250 µg kg⁻¹) and extraction was performed with PBST as reported by Shelby et al. [21]. To avoid matrix effects, matrix-matched calibration with ergometrine was performed in 1:5 diluted flour extract. The calibrants and the diluted extracts of the spiked rye flour samples were analyzed by the magnetic bead-based assay and the detection was performed amperometrically in our flow injection system with the handheld potentiostat as described above. To improve the portability of the system, the detection was performed with a smartphone connected via Bluetooth to the potentiostat. For verification of the results, additional optical detection has been performed for each sample. Fig. 6 shows the recovery rates of the spiked samples obtained with the magnetic bead-based assay with either electrochemical or optical detection. The obtained recovery rates range from 37% to 51%.

Again, it could be demonstrated that optical and electrochemical results are in good correlation. As a screening tool for ergometrine, the developed method could make an important contribution to the detection of ergot alkaloids in the future. The European legal limit for the sum of the 12 major ergot alkaloids is 500 µg kg⁻¹ (and 250 µg kg⁻¹ after July 1, 2024). Thus, the magnetic bead-based immunoassay might facilitate to determine the ergometrine proportion of the total ergot alkaloid content in food products. This may help in the future to directly remove highly contaminated batches of grain from the production facility in mills, before performing precise but expensive and time-consuming analysis by chromatographic methods. By avoiding the storage of contaminated grain for several days, high costs can be avoided, since storage capacities can be used optimally. Thus, the developed method is quite promising for its application in food industry, as only simple and inexpensive equipment is required. Mills usually have some sort of small laboratory at the production site where the quality of the grain is checked upon arrival. Here, several bench-top devices are applied to evaluate, for example, the baking properties or the protein content of the grain. Therefore, a small bench-top flow injection analysis system could be easily established.

However, the development of new, highly specific antibodies against all the other major ergot alkaloids or a rather unselective antibody that is able to return a proxy for a sum parameter might increase the potential of the developed method in the future. Furthermore, the recovery rates might be improved by optimizing the extraction procedure and possibly including a clean-up step. It was reported that the solvent can influence the stability and degree of epimerization of ergot alkaloids and that aprotic solvents, such as acetonitrile are favorable to prevent epimerization [10]. As reported by Köppen et al. epimer-specific determination of ergot alkaloids is important to identify their natural composition in a sample and could be achieved for 12 ergot alkaloids including ergometrine/-inine by using acetonitrile/water (84:16, v:v) for extraction [15]. However, for the developed magnetic bead-based immunoassay, acetonitrile was not used for extraction, as it was observed by ELISA that the sensitivity of the assay in the presence of 5% acetonitrile decreases (see Fig. S4). Still, we think that the obtained recovery rates, together with the relative abundance of ergometrine in

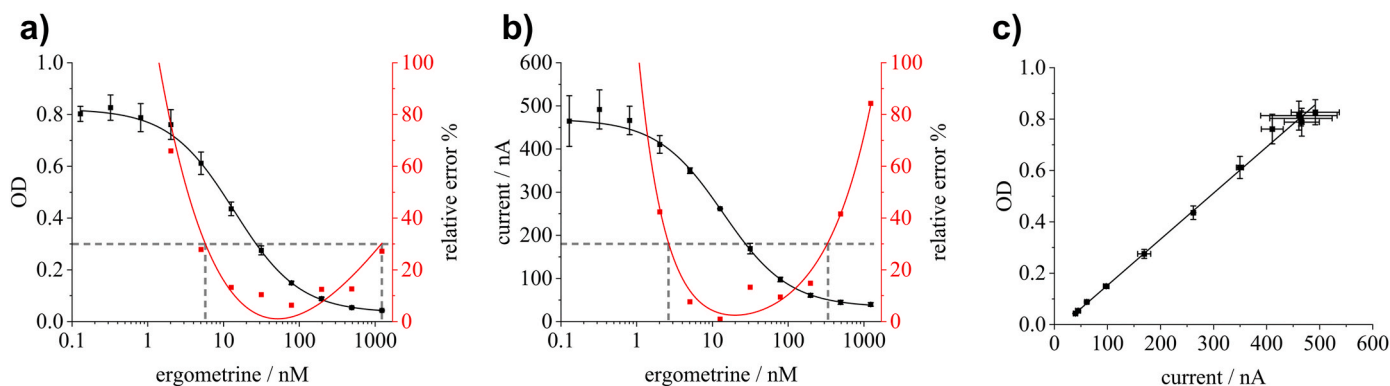


Fig. 5. Calibration curves of the magnetic bead-based immunoassay for ergometrine with a) optical and b) amperometric read-out and their corresponding precision profiles, indicating the relative error of the concentration determination. The LODs were calculated allowing an error of max. 30%, indicated by the grey dashed lines. For the optical read-out, a working range from 6 nM ($2 \mu\text{g L}^{-1}$) to 1.2 μM ($400 \mu\text{g L}^{-1}$) and a C-value of $13.8 \pm 0.7 \text{ nM}$ ($4.5 \pm 0.2 \mu\text{g L}^{-1}$) were obtained. For the calibration curve with amperometric read-out, a working range from 3 nM ($1 \mu\text{g L}^{-1}$) to 300 nM ($100 \mu\text{g L}^{-1}$) and a C-value of $13.8 \pm 0.5 \text{ nM}$ ($4.5 \pm 0.17 \mu\text{g L}^{-1}$) were determined. c) Correlation of optical and amperometric signal ($y = 0.00179x - 0.02652$, $R^2 = 0.99858$).

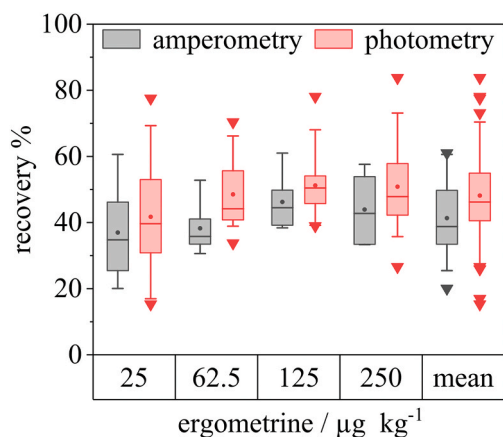


Fig. 6. Recovery rates of the magnetic bead-based assay obtained with electrochemical or optical read-out for ergometrine spiked rye flour samples containing $25 \mu\text{g kg}^{-1}$, $62.5 \mu\text{g kg}^{-1}$, $125 \mu\text{g kg}^{-1}$, and $250 \mu\text{g kg}^{-1}$. For each concentration two independent samples were spiked, and each sample was analyzed in 3 replicates by amperometric detection ($n = 6$) and in 12 replicates by optical detection ($n = 24$). Whiskers represent the interval of 5–95% of all data, dots represent the arithmetic mean and triangles represent the outliers.

complex samples, should be sufficient in their use as a screening method.

4. Conclusions

In summary, a magnetic bead-based immunoassay for ergometrine with portable electrochemical detection has been developed and tested in rye flour. Therefore, the newly conceived tracer consisting of a lysergic acid mimic, a polyethylene glycol (PEG) spacer with 8 units, and a terminating biotin has been used. Fully oxidized TMB has been detected amperometrically in a flow injection system with a custom-made wall-jet flow cell. It could be shown by the example of potassium ferricyanide that the redox current signal intensity obtained in amperometric measurements in a wall-jet flow cell can be increased by increasing the flow rate, which results also in a shorter measuring time. The results obtained with the magnetic bead-based immunoassay by amperometric detection were all verified by optical detection. It could be demonstrated that the electrochemical detection of TMB is a reliable alternative to optical read-out in immunoassays. Furthermore, the magnetic bead-based immunoassay provides a quick alternative to the classical ELISA, as no further blocking of the bead-surface or overnight incubation is required. Ergometrine could be quantified down to 3 nM

($1 \mu\text{g L}^{-1}$) under optimized assay conditions and from 25 to $250 \mu\text{g kg}^{-1}$ in spiked rye flour samples. The detection was performed with a hand-held potentiostat connected via Bluetooth to a smartphone, which offers great promise in meeting the demand for inexpensive devices for on-site food control. To the best of our knowledge, neither a magnetic bead-based immunoassay for ergometrine nor an electrochemical method for ergometrine detection has been reported so far.

Credit author statement

Soraya Höfs: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. Valerie Jaut: Methodology, Investigation, Writing – review & editing. Rudolf J. Schneider: Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2022.124172>.

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