Comparison of On-Line and Off-Line Methods to Quantify Reactive Oxygen Species (ROS) in Atmospheric Aerosols

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Abstract

Atmospheric aerosol particle concentrations have been linked with a wide range of pulmonary and cardio-vascular diseases but the particle properties responsible for these negative health effects are largely unknown. It is often speculated that reactive oxygen species (ROS) present in atmospheric particles lead to oxidative stress in, and ultimately disease of, the human lung. The quantification of ROS is highly challenging because some ROS components such as radicals are highly reactive and therefore short-lived. Thus, fast analysis methods are likely advantageous over methods with a long delay between aerosol sampling and ROS analysis. We present for the first time a detailed comparison of conventional off-line and fast on-line methods to quantify ROS in organic aerosols. For this comparison a new and fast on-line instrument was built and characterized to quantify ROS in aerosol particles with high sensitivity and a limit of detection of 4 nmol H_2O_2 equivalents per m^3 air. ROS concentrations are measured with a time resolution of approximately 15 minutes, which allows the tracking of fast changing atmospheric conditions. The comparison of the off-line and on-line method shows that, in oxidized organic model aerosol particles, the majority of ROS have a very short lifetime of a few minutes whereas a small fraction is stable for a day or longer. This indicates that off-line techniques, where there is often a delay of hours to days between particle collection and ROS analysis, may severely underestimate true ROS concentrations and that fast on-line techniques are necessary for a reliable ROS quantification in atmospheric aerosol particles and a meaningful correlation with health outcomes.

Keywords: organic aerosol; health effects; on-line analysis

1 Introduction

Aerosol particles have long been associated with adverse health effects in the population (Dockery et al., 1993) and are a major public health issue. High atmospheric particle concentrations have been linked in epidemiological studies with e.g., asthma, chronic obstructive pulmonary disease, cardio vascular

diseases and overall mortality (Brunekreef and Holgate, 2002). Many efforts have been made to link these health effects to specific aerosol properties. Studies have focused on physical particle properties such as mass, number concentration and surface area as well as on the metal fraction or on specific organic compounds such as polycyclic aromatic hydrocarbons but so far no particle property could be clearly and consistently linked with the above-mentioned health effects (de Kok et al., 2006, Gerlofs-Nijland et al. 2007). Potential links between the organic fraction of aerosol particles and health effects have hardly been investigated. This may be in part due to the complexity of the organic particle composition and the subsequent poor understanding of which components might be most relevant for the organic fraction's health impact, and in part due to the lack of continuous long-term records of organic aerosol particle composition. The organic fraction often makes up more than 50 % of the aerosol mass and therefore a more detailed analysis of health-relevant organic particle properties is urgently needed.

Many studies hypothesise that oxidizing components in aerosol particles cause oxidative stress in the lung, which may eventually lead to inflammation and disease (Dellinger et al., 2001; Donaldson et al., 2003; MacNee and Donaldson, 2003). Oxidative stress is defined here as a disturbance in the pro-oxidant (i.e., aerosol component) – anti-oxidant balance in favour of the former leading to potential biomolecular damage (Halliwell and Gutteridge, 2007). Particle components which cause oxidative stress are often defined as Reactive Oxygen Species (ROS), and potentially include a wide range of inorganic and organic compounds such as transition metals, hydrogen peroxide (H_2O_2), radicals (e.g., $OH^{\bullet}$, $O_2^{\bullet-}$), and organic (hydro-) peroxides. A number of studies have linked ROS present in organic aerosol to observed health effects (e.g., Pryor and Church, 1991, Donaldson et al., 2003).

There have been many studies that use adapted biological assays to study the ROS concentration of ambient and laboratory generated aerosol. One of the most popular assays uses the fluorescence probe 2'7'-dichlorofluorescein (DCFH), which is reactive to a range of ROS and, which is used in combination with

horseradish peroxidase (HRP). HRP is a redox enzyme that reacts mainly with hydrogen peroxide and organic hydroperoxides (Berglund et al., 2002; Furtmüller et al., 2000; Cathcart et al., 1983). The reactivity of HRP towards other ROS is not very well described but a range of ROS components such as Criegee radicals and peroxy radicals can form hydrogenperoxides in an aqueous solution (Pryor and Church, 1991; Wang et al., 2012; Hasson et al., 2001) and can thus be quantified indirectly with the DCFH/HRP assay.

ROS are usually quantified with off-line techniques in extracts of aerosol particles collected on filters (Hung and Wang, 2001; Venkatachari et al., 2005). Due to the reactive nature of ROS, such off-line methods risk severely underestimating the true ROS concentration in particles because some ROS components (e.g., radicals or peroxides) might decompose during sample collection and work-up. Therefore, two recent studies presented on-line and semi on-line techniques to quantify particle-bound ROS with DCFH with the aim to reduce the time between collection and analysis associated with filter collection and for higher time resolution measurements (Wang et al., 2011; King and Weber, 2013).

In this study we present a new on-line instrument further developing the concept of Wang et al. (2011) that uses a continuous flow version of the DCFH/HRP assay for the quantification of particle-bound ROS. The instrument is designed to allow for a very gentle and fast ROS extraction to minimize ROS decomposition before analysis. We present a characterisation of the instrument performance and present for the first time a detailed comparison between off-line filter collection methods and the on-line automated system for ROS quantification to establish the advantages of on-line measurements. Results clearly show that the major fraction of ROS in oxidized organic aerosol particles has a very short lifetime emphasizing the need for fast on-line ROS analysis techniques.

2 Experimental

At the core of the instrument described here is the aqueous phase reaction cycle of ROS with horseradish peroxidase (HRP) and 2'7'-dichlorofluorescein (DCFH) leading to the fluorescent reaction product DCF, which is ultimately quantified and related to the ROS concentration in the aerosol particles. The general reaction mechanism between ROS, HRP and DCFH is described below. This is followed by a description of the model aerosol particle generation, the new on-line instrument, a corresponding off-line method, and the comparison between these two methods.

2.1 DCFH Assay

Liquid phase ROS concentrations were quantified using the fluorescence probe DCFH in combination with the peroxidase enzyme catalyst HRP. As shown in Figure 1a, H_2O_2 (also representing other ROS) reacts with HRP to form an intermediate which subsequently oxidises two equivalents of DCFH to the fluorescent product DCF (Berglund et al., 2002). The concentration of the product DCF is measured using fluorescence spectroscopy (excitation at 470 nm and emission at 520 nm). HRP is reactive towards hydrogen peroxide, organic hydroperoxides (Cathcart et al., 1983) and, as mentioned above, possibly other ROS components as part of the peroxidase cycle.

Background steady state concentrations of H_2O_2 at concentration levels of up to 60 nmol dm^{-3} are present in laboratory grade deionised water as it is in equilibrium with dissolved oxygen giving rise to an unavoidable background fluorescence signal (Hwang and Dasgupta, 1986; Venkatachari and Hopke, 2008). In addition, dissolved oxygen can also react with HRP to oxidise DCFH to DCF in the oxidase cycle as shown in Figure 1b. Both the background concentrations of H_2O_2 and reaction with oxygen can be accounted for by the subtraction of blank measurements. Previous studies have attempted to reduce the background by removal of H_2O_2 using metal catalysts such as MnO, however, once removed, steady state concentrations of H_2O_2 re-establish rapidly, a process which is accelerated by visible and long wave UV light (Venkatachari and Hopke, 2008). To reduce the variability in background H_2O_2 concentrations, and to prevent photo-oxidation of DCFH, the assay was carried out under dark

conditions. H_2O_2 concentrations in the blank are in the same order of magnitude as the ROS in aerosol particles (see below). Thus, a careful characterisation and subtraction of the blank, especially its stability over time, is necessary for reliable results. In this study the assay is calibrated using H_2O_2 and all results are given as an equivalent H_2O_2 concentration.

The fluorescent probe DCFH is very reactive and prone to decomposition and is therefore synthesised each day it is used. It is synthesised from the stable di-acetate form DCFH-DA. For a 250 ml 10 μM DCFH solution, a stock solution of DCFH-DA (Sigma Aldrich) in methanol (1.218 ml, 1.0 mg ml^{-1}) is reacted with sodium hydroxide (10 ml, 0.01 mol dm^{-3}) for 30 min under dark conditions. To make a pH 7.2 buffered solution and neutralise any remaining sodium hydroxide, phosphate buffered saline (PBS) (Sigma Aldrich) is added (25 ml, 1.0 mol dm^{-3}) to make a final solution of 10 μmol dm^{-3} DCFH in 0.1 mol dm^{-3} PBS. Horseradish peroxidase (HRP) (Type VI, Sigma Aldrich) solutions are made up in HPLC water (Rathburn) and 10 % PBS.
The DCF detection system is described below in 2.3 and 2.4.

2.2 Aerosol Generation

Oleic acid aerosol particles were generated and oxidized using the apparatus shown in Figure 2. To characterize the instrument developed here oxidized oleic acid was chosen as a model of aged organic aerosol which has been well characterised in earlier studies (Vesna et al., 2009; Ziemann, 2005). Oleic acid particles are ideal for such a calibration because the oxidation products are reasonably well understood and include a range of different functional groups that reflect potentially health-relevant compound classes present in atmospheric aerosol. This specifically includes peroxides, hydroperoxides, stabilized Criegee radical intermediates, as well as organic acids and aldehydes (Ziemann, 2005).

Oleic acid aerosol particles were generated by heating approximately 2.0 ml of pure oleic acid (Reagent Grade 99 %, Sigma Aldrich) in a schlenk flask contained in a heated aluminium block (100-110°C) (Figure 2). Nitrogen was flowed through the flask at 0.5 L min^{-1}. Oleic acid particles were formed through

homogenous nucleation downstream of the flask as the vapour cooled to room temperature. Ozone gas was generated by flowing air, at 1 L min^{-1}, past a 185 nm UV light source (Appleton Woods). To simulate aged (oxidized) aerosol particles the ozone and particle flows were combined and passed through a 2 L glass tube, giving a reaction time of approximately 80 seconds, and an ozone concentration of 130 ppm. The ozone concentration was measured using a commercial ozone analyzer (49i, Thermo Scientific). The oxidized aerosol was then passed through an activated charcoal denuder to remove excess ozone and organic vapours. Ozone concentrations were regularly measured after the denuder to assure that all ozone was removed by the denuder. Then the aerosol was diluted with nitrogen to make a total flow of 6 L min^{-1}. Experiments were carried out at room temperature (~ 20 ^{o}C) and at low relative humidity (< 2 % RH).

Particle size distributions were measured using a Scanning Mobility Particle Sizer (SMPS): a TSI model 3081 differential mobility analyser (DMA) combined with a model 3775 condensation particle counter (CPC). The SMPS operated at a sampling rate of 0.3 L min^{-1} and a DMA sheath flow of 3 L min^{-1} giving a measurable particle size range of 14 – 700 nm. Particle number size distribution data was acquired using TSI AIM software. The density of the oxidized particles was assumed to be 1.0 g cm^{-3} as taken from previous studies of oxidised organic acid particles (Katrib et al., 2005).

Aerosol mass concentrations were controlled through the oleic acid heating temperature. The temperature was varied between 100 – 110 ^{o}C and could be maintained with a variability of ± 0.5 ^{o}C. This corresponded to a stable and adjustable oleic acid aerosol particle mass concentrations of 10 – 200 μg m^{-3} with a variability of typically less than ± 10 % over a period of 1 hour and with a particle size mode of 93 ± 5 nm throughout the entire mass range.

2.3 On-line Measurements

Figure 3 shows a schematic of the on-line instrument for ROS quantification, which combines in a continuous flow system an efficient particle collection with the DCFH/HRP assay described above.

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215 Air is pumped through the particle collector (Takeuchi et al., 2005) at a flow rate
216 of 5 L min^{-1} where it intersects with the aqueous HRP collection solution (1 unit
217 ml^{-1}, 10 % PBS) pumped at 1 ml min^{-1} using a peristaltic pump (Watson Marlow
218 323 with 318C pump head) . This creates a fine spray of the collection solution.
219 The spray serves two functions: to uniformly wet the filters used for collection of
220 particles and to allow direct collection of particles through collision with the
221 spray droplets within a fraction of a second of entering the instrument. If not
222 collected in the spray, particles come into contact with the HRP solution on the
223 filters and their water-soluble fraction is extracted into the solution where ROS
224 can react with HRP. Therefore it is difficult to define an exact time between
225 particles entering the instrument and the reaction of extracted ROS components
226 with HRP but due to the compact design of the particle collector it is estimated
227 that this is on a time scale of seconds to a few minutes. The filters consist of a
228 paper filter (25 mm, Whatman Type 1) on top of a hydrophilic support filter (25
229 mm, Millipore, Isopore™, 5.0 μm). The collection efficiency is greater than 95 % for
230 particles with an aerodynamic diameter > 100 nm, and drops to 50 % at 50 nm.
231 The solution is collected in the base of the particle collector, from where it is
232 pumped out at 1 ml min^{-1}. The particle extract/HRP solution is then mixed with
233 DCFH solution (10 μmol L^{-1}, 10 % PBS) also being pumped at 1 ml min^{-1}. This
234 mixing dilutes both reactants to the same concentrations of HRP (0.5 unit ml^{-1})
235 and DCFH (5 μmol L^{-1}) used in the off-line method described later.
236 The effect of HRP concentrations higher than 0.5 unit ml^{-1} was tested. While this
237 resulted in an increased reaction rate with ROS it also increased the background
238 reactions described in Figure 1b and thus did not result in an overall better
239 sensitivity of the assay.
240 The combined solution then flows through a 20 ml Teflon tubing (3.175 mm OD,
241 1.5 mm ID) reaction coil, immersed in a water bath at 40°C, giving a reaction
242 time of approximately 10 minutes in the water bath. The total residence time of
243 the reagents in the flow system (from the point of mixing to the fluorescence
244 measurement) is approximately 15 minutes, shown in off-line studies to allow
245 for complete reaction of the assay with H_2O_2 concentrations up to 800 nmol dm^{-3}
246 (as shown later, Figure 6a). The solution is then passed through a quartz

fluorescence flow cell with a 1 cm^2 cross-section and a 3 cm^3 volume. Due to inhomogeneous mixing, the mixing time in the fluorescence flow cell is approximately 15 minutes, limiting the time resolution of measurements. The cell is mounted vertically, with the solution entering at the base, allowing for gas bubbles in the system to rise quickly to the top of the cell without affecting fluorescence measurements. The fluorescence is measured using a temperature stabilised LED light source (470 nm, Luxeon V Star) coupled with an Ocean Optics USB2000+ spectrometer recording the fluorescence emission of DCF at 520 nm. The fluorescence data is acquired and analysed using LabView (National Instruments, version 8.6). Between successive measurements the instrument can be cleaned by replacing the aerosol flow with a clean flow of synthetic air till ROS concentrations reach blank levels.

This on-line instrument differs from previously described instruments mainly in the particle collection process. The only other on-line instrument described in the literature (Venkatachari and Hopke, 2008; Wang et al., 2011) uses a particle into liquid sampler (PILS)(Orsini et al., 2003), which allows particle collection at high flow rates (15 L min^{-1}) but uses a high temperature steam to grow particles for collection: these high temperatures may influence analysis of the sample due to the unstable nature of some ROS. In the instrument described in this study, ROS-containing particles are mixed and extracted with the HRP solution at room temperature.

In another system recently described (King and Weber, 2013) samples are collected for a time period of five minutes before analysis using the DCFH/HRP assay. This short delay of five minutes before analysis may lead to loss of reactive components (see comparison of on-line and off-line method below).

2.4 Off-line Measurements

Off-line measurements of the fluorescence response of the DCFH/HRP assay with H_2O_2 were used to optimize operation conditions of the on-line instrument and to compare the performance of on-line and off-line quantification methods. Solutions of known H_2O_2 concentration (0.8 ml) were combined with DCFH (1 ml, 10 μM, 20 % PBS) and HRP solution (0.2 ml, 5 unit ml^{-1}) in a disposable UV-

cuvette (Brand, semi-micro, Sigma Aldrich), giving final concentrations of HRP and DCFH as used in the on-line measurements. Fluorescence measurements were taken continuously during a 15-minute reaction time using a 4-way cuvette holder (Ocean Optics), which was maintained at 40 ^{o}C by passing heated water through its base. The 15-minute reaction time allows for complete reaction (see discussion below and Figure 6a) and is equivalent of the reaction time in the on-line instrument. The same light source, spectrometer, and software were used as for the on-line method.

In addition to H_2O_2 calibration solutions, ROS concentrations were also quantified in oxidized oleic acid particles with the off-line method and compared with the on-line instrument. For this comparison aerosol samples were collected on Teflon filters (Millipore, Durapore, 0.1 μm, 47 mm, Sigma Aldrich) at a flow rate of 5 L min^{-1}. The filters were collected for a range of times from 1 – 15 minutes. To determine the total particle mass on the filters the aerosol size distribution at the time of collection was measured using a SMPS, with a typical mass concentration of 100 - 200 μg m^{-3}. Following collection, a 14 mm diameter punch of the filter was extracted in water (1 ml, HPLC grade, Rathburn). Two different methods were compared for extraction, firstly sonication for 15 minutes in an ultrasonic bath (Grant), and secondly vortexing for 3 minutes in a vortex mixer (Fisher-Scientific). The 3-minute time for vortexing was established as the time for maximum yield whilst analysing the samples as fast as possible. The aqueous extract (0.8 ml) was combined with DCFH (1ml, 10 μM, 20 % PBS) and HRP solution (0.2 ml, 5 unit ml^{-1}) in a disposable UV cuvette and analysed as described above for H_2O_2 calibration solutions. Blank filter measurements were taken for each particle collection time by placing a HEPA filter in front of the collection filter and these were subtracted from the equivalent sample measurements.

3 Results and Discussion

3.1 Online Measurements

312 The sensitivity of the on-line instrument was determined with H_2O_2 standard
313 calibration solutions. For this the particle collector was by-passed and reactants
314 were pumped directly out of their respective reservoirs to the heated reaction
315 coil. Known H_2O_2 concentrations were included in the DCFH stock for calibration
316 as this mixture is un-reactive without HRP. The fluorescence of the reaction
317 mixture was measured after a 15-minute reaction time. Concentrations of
318 reactants in the flow system were as described above (i.e., 0.5 unit ml^{-1} and 5
319 μmol dm^{-3} for HRP and DCFH, respectively). The calibration results are shown in
320 Figure 4, showing a strong linear relationship between the fluorescence intensity
321 and H_2O_2 concentration up to 200 nmol dm^{-3}. The limit of detection (LOD) of
322 H_2O_2 in the liquid phase is approximately 10 nmol dm^{-3} and is derived from the
323 variability (three standard deviations) of the blank signal intensity, taken on the
324 same day. Absolute fluorescence intensities of the blank were approximately
325 2000 counts and the data shown in Figure 4 is blank subtracted. Blank
326 measurements of the solvent are taken at least once a day. This LOD equates to
327 an aerosol phase LOD of approximately 4 nmol H_2O_2 m^{-3}, based on a particle
328 extract/HRP flow rate of 1 ml min^{-1}, a gas sampling rate though the particle
329 collector of 5 L min^{-1} and a flow system liquid flow rate of 2 ml min^{-1} with a
330 linear range from 4 to at least 80-100 nmol H_2O_2 m^{-3}. This detection limit should
331 be sufficient for ambient atmosphere measurements where often ROS
332 concentrations of around 5 nmol m^{-3} were reported (Venkatachari et al., 2005;
333 Wang et al., 2011 and references therein). King and Weber (2013) measured
334 lower ROS concentrations with an average of 0.16 nmol H_2O_2 m^{-3}, which would
335 be below the LOD of our current instrument version.
336 The ROS concentrations detected and reported below for the oxidized oleic acid
337 aerosol particles are given as equivalent H_2O_2 concentrations.
338
339 To characterize the instrument further, and with real aerosol particles, the ROS
340 concentration was quantified in oxidized oleic acid particles. Oleic acid particles
341 were generated as described above and particle-bound ROS concentrations were
342 measured over a range of aerosol mass concentrations (10 – 170 μg m^{-3}) to
343 generate a calibration curve including the particle collector and thus assessing
344 the linearity of the instrument response considering all parts of the on-line

345 instrument. The particle size distribution was measured using an SMPS and the
346 modal size (93 ± 5 nm) was found not to change for the mass concentrations
347 range considered here. Comparison of the collection efficiency of the particle
348 collector with the generated aerosol size distribution showed that the particle
349 collector was able to sample more than 90 % of the aerosol mass.
350
351 On-line measurements shown in Figure 5 were taken over three separate days
352 and only small day-to-day variation was observed. Blank measurements, taken
353 by placing a HEPA filter in front of the particle collector, were subtracted from
354 sample measurements. The aerosol generation technique was repeatable and
355 generated particles with a consistent ROS concentration per particle mass for a
356 wide range of mass concentrations. Thus, oxidized oleic acid can be used as a
357 true, easy to use, and reliable aerosol standard for ROS.
358
359 A strong correlation between the total particle mass and the ROS concentration
360 is observed (Figure 5). The linear range of the aerosol calibration extends over a
361 similar range as the calibration with H_2O_2 standard solutions shown in Figure 4,
362 i.e., from ~20 to ≥ 100 nmol H_2O_2 m^{-3}. If a linear fit is applied to the data, the
363 gradient indicates a concentration of 0.58 ± 0.07 nmol ROS (i.e., H_2O_2
364 equivalents) per μg of oxidized oleic acid particle mass.
365
366 **3.2 Off-line Measurements**
367 The off-line method was calibrated using standard solutions of H_2O_2 up to 800
368 nmol dm^{-3}. The calibration curve is shown in Figure 6. Graph (a) shows the
369 increase in fluorescence of the reaction mixture over a 15-minute period for
370 different H_2O_2 concentrations. It can be seen that the initial rate is independent
371 of the H_2O_2 concentration and after 5 – 10 minutes (depending on the H_2O_2
372 concentration) the increase in fluorescence is minimal and this is deemed to be
373 when all H_2O_2 present has reacted. A 15-minute reaction time was therefore also
374 used for the on-line instrument. Graph (b) plots the fluorescence for H_2O_2
375 standards up to 800 nmol dm^{-3} after the 15-minute reaction time when reaction
376 is complete as indicated with a dotted line in graph (a). In both graphs the blank
377 signal for the HPLC water used has been subtracted. The results indicate a

degree of non-linearity in the assay response in the off-line method at high H_2O_2 concentrations. However, in the region of interest, up to 200 – 400 nmol H_2O_2 dm^{-3}, the relationship between H_2O_2 concentration and fluorescence intensity is approximately linear. The liquid phase limit of detection is derived from the variation in H_2O_2 concentrations present in the water used as a solvent. Background H_2O_2 concentrations present in the water used for extraction ranged from 40 – 90 nmol dm^{-3}, however, on a given day the background concentrations were more stable with a variation of less than ± 10 nmol H_2O_2 dm^{-3}, comparable to the on-line instrument. The limit of detection of particle-bound ROS (in nmol m^{-3} air) is dependent on the air volume collected. Assuming a constant aerosol mass concentration, the LOD decreases with increasing collection time. For a one-minute filter collection (air flow: 5L min^{-1}) of oleic acid aerosol with a mass concentration of 125 μg m^{-3}, this equates to a detection limit of approximately 0.36 nmol H_2O_2 μg^{-1}. For a 10–minute filter collection, the LOD decreases to 0.036 nmol H_2O_2 μg^{-1} (see discussion below for Figure 7).

For the off-line ROS quantification in oxidized oleic acid particles two filter extraction methods were compared, firstly extraction by sonication and secondly extraction by vortexing. Extraction by sonication for 15 minutes gave results where the blank filter samples showed higher ROS concentration than the sample filters on which the oxidised particles were collected. Sonication of pure HPLC water gave ROS concentrations of 150 nmol dm^{-3}, showing an increase of 80 nmol dm^{-3} compared to the same water sample before sonication, measured at 70 nmol dm^{-3}. The sonication of water with dissolved air present is known to create hydroxyl radicals due to the high temperature and pressure created by the collapse of bubbles formed by cavitation. Two hydroxyl radicals then react to create H_2O_2 (Mark et al., 1998). When sample filters are sonicated, the higher concentrations of soluble organics will likely scavenge hydroxyl radials formed by cavitation and thus reduce the amount of H_2O_2 produced, which might explain why sonicated blank filters showed higher ROS concentrations. This emphasises that sample extraction procedures should be carefully characterized when ROS concentrations are determined.

As sonication itself was shown to be creating H_2O_2 during the extraction process, only vortex extraction was used for the comparison of the off-line and on-line method. This extraction process is still more vigorous than the process involved in the on-line analysis and would be expected to result in an equally quantitative extraction efficiency as the on-line method.

3.3 Comparison of on-line versus off-line methods

For a comparison of the off-line and on-line technique, measurements were performed at oleic acid aerosol mass concentrations of approximately 125 μg m^{-3} which is well within the linear range of both the on-line and off-line calibration curves. The filter collection time was varied from 1 – 15 minutes and was repeated at least three times. As the sampling time for the off-line analysis was increased, and thus the delay between the start of particle collection and the addition of HRP and DCFH (i.e., the start of the off-line analysis) was increased, the ROS measured per particle mass decreased exponentially. In Figure 7, the measurements shown by the filled black circles are taken directly after sampling, the measurements shown by the open circles are for filters collected for 15 minutes and stored in a closed vial in the dark at room temperature for approximately 7 hours and 22 hours before the remainder of the same filter was extracted and analysed. When measured immediately after a short collection time (1 minute), off-line measurements gave a ROS concentration of 0.75 ± 0.40 nmol H_2O_2 μg^{-1} oleic acid. The relatively large error is mainly due to the very short particle collection time of one minute, which inherently introduces uncertainties in the total collected particle mass. However, after a 15 minute sampling time ROS concentrations fell to 0.14 ± 0.05 nmol H_2O_2 μg^{-1}. When the filters collected for 15 minutes were reanalysed hours later, no further decrease in the ROS concentration was observed.

These off-line results can be directly compared to measurements taken using the on-line instrument. The calibration curve in Figure 5 shows that a ROS concentration of 0.58 nmol H_2O_2 μg^{-1} oleic acid is measured with the on-line instrument (derived from the slope of the regression analysis in Figure 5). This is

slightly lower but well within error limits of the shortest off-line analysis data point with 0.75 ± 0.40 nmol H_2O_2 μg^{-1} as shown in Figure 7.

This comparison between on-line and off-line analysis clearly shows that the ROS concentration in organic particles decreases strongly within 10 – 15 minutes after the collection on a filter, with a major fraction of ROS having an half-life of a only few minutes.

These results suggest that there are two separate types of ROS in oxidized oleic acid particles: short-lived species with a half-life of a few minutes; and long-lived species that are stable for hours to days. The short-lived ROS are present in much higher concentrations than the long-lived ROS and could include organic radical species such as the stabilised Criegee intermediate. The measured long-living ROS could potentially be organic hydroperoxides.

The observed half-life of a few minutes for the short-lived species is much shorter than that suggested by the only previous study aiming to estimate the lifetime of ROS concentration in oxidized organic aerosols, which determined a half-life of six hours (Chen et al., 2011). Our results also clearly contrast the on-line/off-line comparisons of Wang et al (2011) and King and Weber (2013) where comparable ROS concentrations were measured with on-line and off-line methods. This discrepancy could possibly be explained by the different analysis methods used and the different aerosols analysed.

4 Conclusions

A new instrument for on-line ROS quantification is presented and characterized. One of the main differences to the only other previously described on-line ROS instrument (Wang et al., 2011) is the gentle particle extraction procedure applied here, which is crucial considering the reactive and short-lived nature of many ROS components. The new on-line instrument has a limit of detection of approximately 4 nmol H_2O_2 equivalents per m^3 air and has a time resolution of

approximately 15 minutes. This allows following fast changing ROS concentrations in the atmosphere or in laboratory studies.

For the first time we present a detailed comparison of on-line with off-line ROS quantification methods using laboratory-generated oxidized oleic acid particles. This comparison clearly shows that ROS in oxidized organic particles can be divided into two fractions, a short-lived fraction with a half-life of only a few minutes, and a long-lived ROS fraction which is stable for hours or days. The total ROS concentration (in H_2O_2 equivalents) is dominated by the short-lived ROS fraction, being approximately 5 times larger than the long-lived ROS components. The comparison of on-line and off-line analyses presented here suggests that off-line analyses methods are likely to severely underestimate particle-bound ROS concentrations, and that fast on-line techniques are crucial to obtain reliable, health-relevant information on ROS concentrations in atmospheric aerosols.

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Figure Captions

Figure 1. The reaction scheme of DCFH/HRP with peroxides (a) demonstrating the initial reaction of ROS (H_2O_2) with HRP, followed by reaction with DCFH to form the fluorescent product DCF. The reaction of HRP with oxygen (b) also leads to the formation of DCF and is accounted for in blank measurements (schematic modified after Berglund et al., 2002).

Figure 2. Schematic of the oxidised oleic acid aerosol generation system where oleic acid aerosol is produced through heating and reacted with ozone before passing through a charcoal denuder to remove organic gas phase products and excess ozone. The number size distribution of the aerosol particles is measured using an SMPS in parallel to particle-bound ROS concentration measurements.

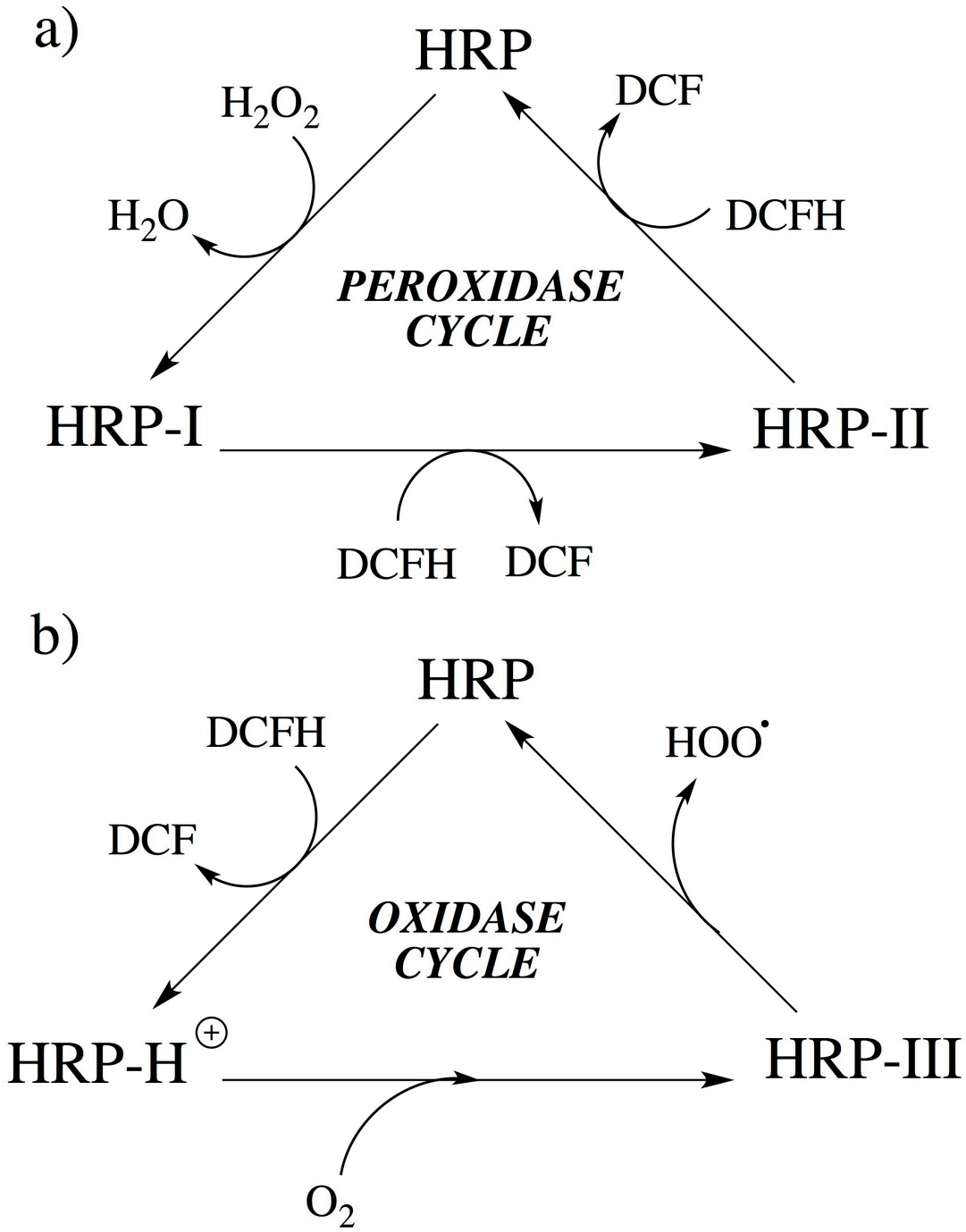
Figure 3. Schematic of the on-line particle-bound ROS measurement instrument. Particles are collected on hydrophilic filters in the small particle collector (150 mm height, 30 mm diameter) and soluble components are extracted for on-line analysis using the DCFH/HRP-fluorescence assay.

Figure 4. Calibration of on-line instrument with hydrogen peroxide, showing a linear relationship to fluorescence intensity and an LOD of approximately 10 nmol H_2O_2 dm^{-3}. Error bars are smaller than the symbol size and thus are not visible.

Figure 5. Results of the on-line instrument showing measured ROS concentrations versus oxidised oleic aerosol mass concentrations. This strong linear relationship shows that the aerosol generated has a consistent ROS concentration per particle mass. Measurements were performed during three days demonstrating the reproducibility of the ROS generation using oxidized oleic acid. Each symbol (squares, circles and diamonds) represents the measurements taken during one of the three days.

Figure 6. Calibration of off-line method with hydrogen peroxide showing the increase in fluorescence intensity with time for a range of hydrogen peroxide concentrations (a) and the end-point fluorescence intensity after 15 minutes against H_2O_2 concentration (b).

Figure 7. Comparison of ROS concentrations in oxidized oleic acid particles using the on-line instrument and the off-line method. Results from the off-line method show a strong decrease in ROS concentration with increased time between filter sampling and analysis, which levels off after ~15 minutes (filled and empty circles). The limit of detection for the off-line method is indicated by the grey striped line. ROS concentrations quantified with the on-line instrument are shown for comparison (diamond symbol) and are comparable with the off-line method only for samples with the shortest collection time (one minute).



633
634
635 Figure 1
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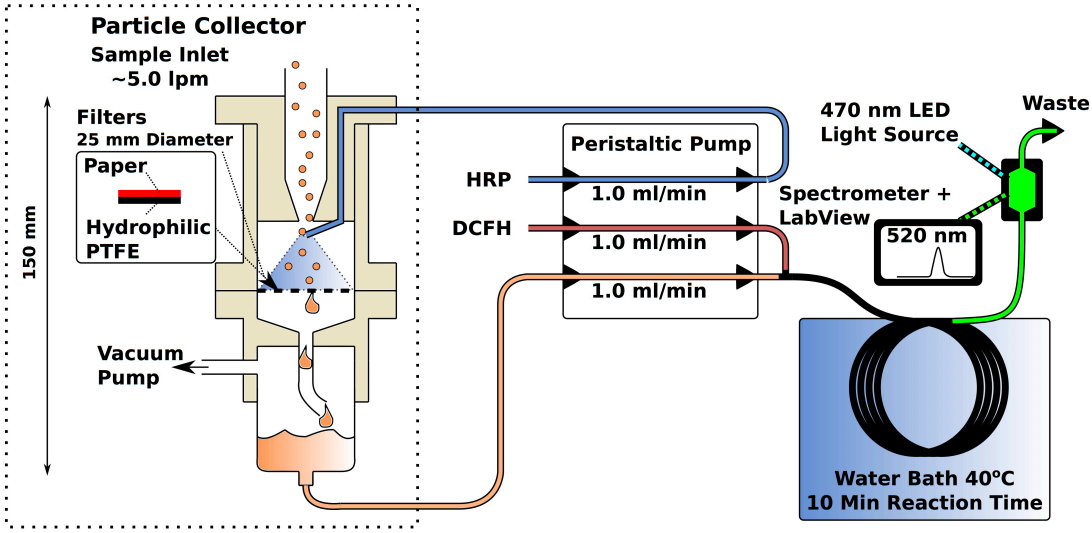


Figure 2

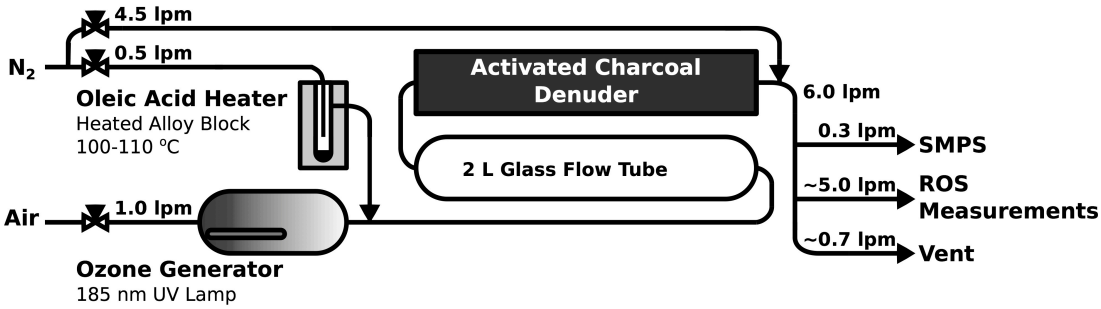


Figure 3

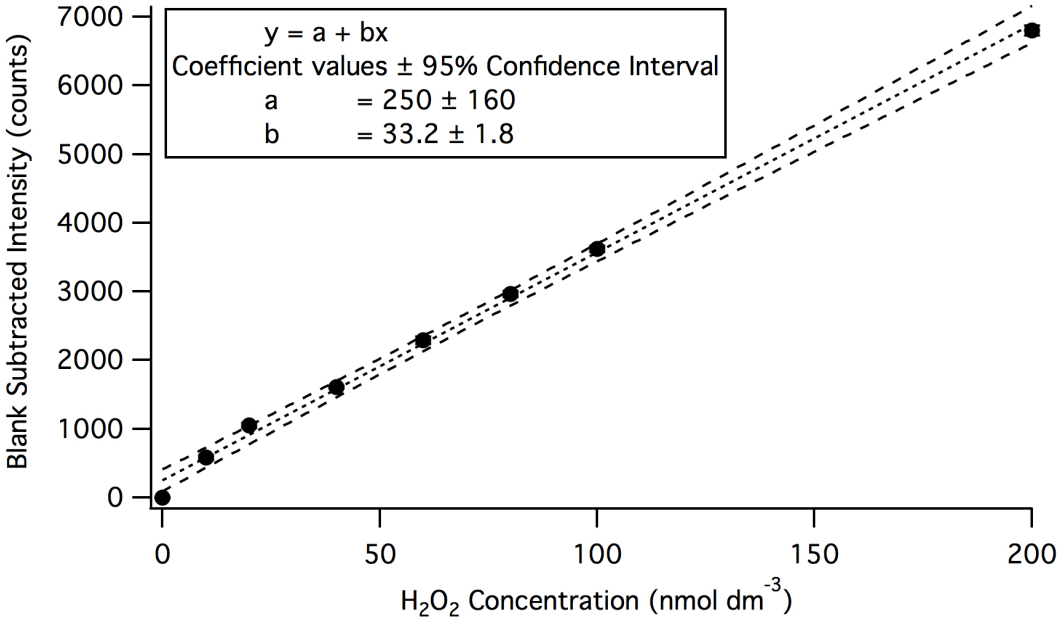


Figure 4

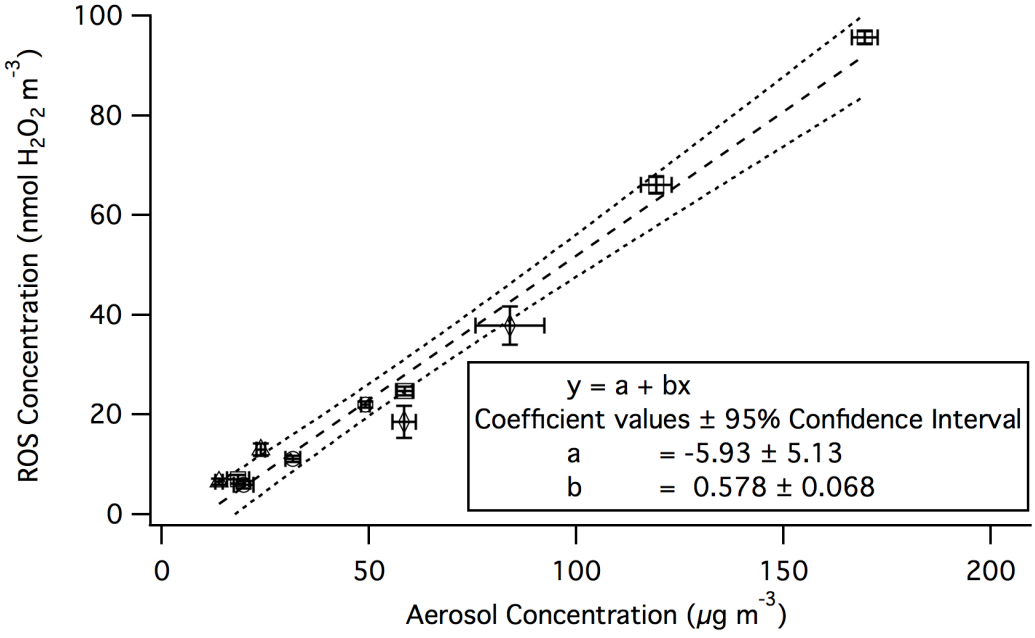
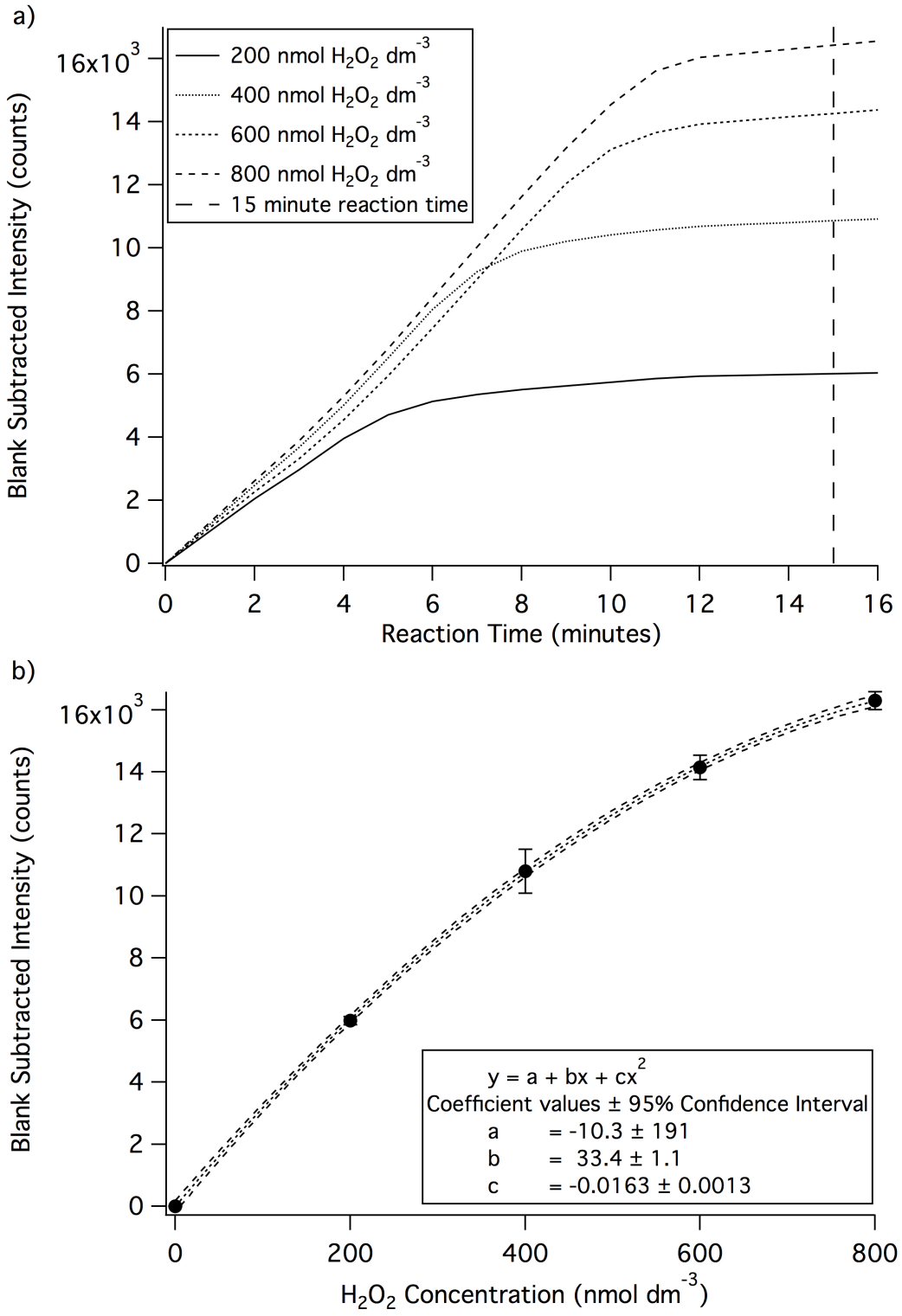


Figure 5



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656 Figure 6

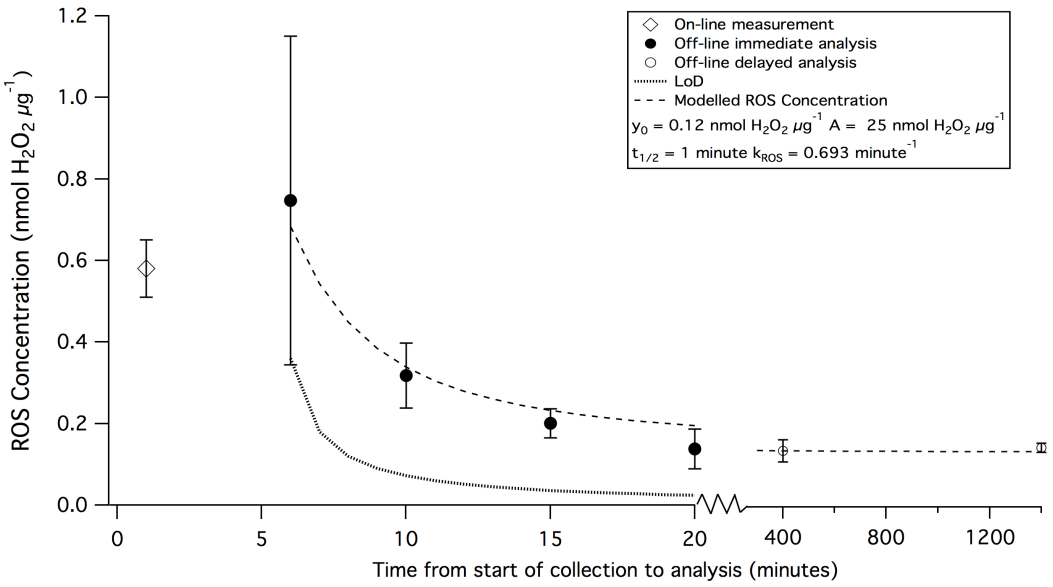
On-line measurement
Off-line immediate analysis
Off-line delayed analysis
LoD
Modelled ROS Concentration
y0 = 0.12 nmol H2O2 µg-1 A = 25 nmol H2O2 µg-1
t1/2 = 1 minute kROS = 0.693 minute-1
ROS Concentration (nmol H2O2 µg-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
400
800
1200
Time from start of collection to analysis (minutes)

Figure 7