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Coupling SFE to uterotonic bioassay: an on-line approach to analysing medicinal plants

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Abstract

Supercritical fluid extraction has been directly coupled on-line to a uterotonic bioassay, using guinea pig uterine smooth muscle in vitro. This technique was developed for the detection of uterotonic compounds present in medicinal plants used during pregnancy to induce or augment labour. The direct passage of CO_2 into the muscle chamber led to adiabatic cooling of the physiological fluid and inhibition of muscle contraction. This was alleviated by the construction of a CO_2 reduction interface together with the passage of carbogen which aided in the rapid displacement of excess CO_2. The on-line system was evaluated with four plants (*Clivia miniata* (Lindl.) Regel, *Ekebergia capensis* Sparrm., *Grewia occidentalis* L. and *Asclepias fruticosa* L.) that are currently used during pregnancy by some black South African women. Extractions were performed with water modified supercritical CO_2. Fractions of supercritical fluid extracts, obtained by sequentially increasing the pressure from 200 to 300 and 400 atm at constant temperature were transferred directly to the muscle chamber to identify the active fractions. The 400 atm extracts of *C. miniata*, *A. fruticosa* and *E. capensis* displayed maximum uterotonic activity while only the 300 atm extract of *G. occidentalis* induced uterine muscle contraction. This technique proved to be a safe and sensitive method for analyzing medicinal plants that contain uterotonic substances hence assisting in rapidly validating the uterotonic properties and detecting any toxic effects of these extracts. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Uterotonic bioassay; Supercritical fluid extraction; Medicinal plants; Guinea pig uterus

1. Introduction

Despite the dramatic advances made in allopathic medicine over the past century, there has been an increasing interest in complimentary health systems [1]. The continent of Africa provides an extremely diverse range of plants that, over the centuries, has found use in treating a wide variety of ailments. Traditional medicine currently still plays an integral part in the lives of most black South Africans. In KwaZulu-Natal, traditional healers are acceptable, accessible and greatly utilised, and hence play an important role in tribal communities. However the quality, safety

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and efficacy of most traditional medicines has not been investigated scientifically.

Ingestion of plant extracts during pregnancy is common and as many as 57 different plants are used [2]. Different concoctions (isishilambezo mixtures) are made to either provide supplements that aid the growth of a foetus or to act as uterotonic agents that facilitate or induce labour. Hence, investigation of components contained in medicinal preparations is important as there may be more than one active ingredient and, unlike conventional pharmaceuticals, chemical composition can vary from batch to batch. Furthermore, it is believed that the consumption of these concoctions can result in foetal meconium staining at delivery [3].

Bioassays are a common adjunct to chemical analysis. Traditionally, samples to be used for bioassay have been prepared by solvent extraction, vacuum distillation, membrane processes, lyophilization, etc [4]. These techniques offer some success but also some significant disadvantages. Supercritical fluid extraction (SFE) has proven to be a valuable alternative method of extraction that has received tremendous attention recently especially in view of increasing environmental concerns about the use of liquid solvents in the extraction of natural products [5–7]. In comparison with classical liquid–solid extraction methods, SFE offers many potential advantages. The favourable mass transport properties of supercritical fluids (i.e. low viscosities and high diffusivities) impart excellent matrix penetrating power to the extractant phase; consequently, extraction times are dramatically reduced compared to liquid based methods. Other advantages include increased selectivity, easier analyte fractionation, and on-line coupling with other analytical techniques [8–10]. Further, by using a fluid such as CO_2 with a relatively low critical temperature (31°C), extractions can be performed under mild thermal conditions. In addition, CO_2 provides an extraction environment free from molecular oxygen, thereby limiting potential oxidation of the extracted solutes [11]. Although there have been reports of SFE coupled to immunoassay analysis, these studies have been performed using off-line collection of the extracted analytes [12,13]. On-line approaches provide potential for combined sample preparation and analysis and the potential to transfer every extracted analyte molecule to the detection system thereby increasing sensitivity. A further advantage is the elimination of sample handling prior to the bioassay hence eliminating the possibility of sample contamination.

Supercritical CO_2, unlike many liquid extraction solvents, is a nontoxic extraction medium making it appropriate for interfacing with the bioassay since the problems of artifactual solvent toxicity inherent in conventional extraction and fractionation schemes is eliminated. Unfortunately, CO_2 is not sufficiently polar to extract highly polar components. Polar compounds therefore show limited solubility in pure CO_2 and in such cases either a more polar supercritical fluid should be used or a modifier added. However, it must be remembered that the addition of modifiers changes the critical point of the mixture from one recorded for the pure fluid. The critical temperature of the mixed solution is higher than that of the pure fluid, and therefore the extraction temperature should be raised to ensure that a single phase supercritical fluid is present during SFE to avoid possible solute partitioning between the two phases that might coexist. Hence it is important to recognise the magnitude of this change so as to adjust the experimental parameters accordingly. Methanol has been by far the most commonly used modifier in a wide range of sample matrices [14–17]. However, methanol is toxic to biological tissue and was not the modifier of choice for this study. Instead, water was used as a modifier and added directly to the matrix. Although water is scarcely soluble in liquid carbon dioxide ($\sim 0.1\%$ m m^{-1} at 20°C), its solubility increases with increasing temperature ($\sim 0.3\%$ m m^{-1} at 50°C), and has thus proven effective in the extraction of caffeine from green coffee beans, where the caffeine content of coffee was reduced from 0.7–3 to 0.02% by this method [18]. The potential of modifiers has also been demonstrated for the extraction of alkaloids thebaine, codeine and morphine from poppy straw [19].

Using the on-line system, the uterotonic effects of four plants viz *Clivia miniata* (Lindl.) Regel., *Ekebergia capensis* Sparrm., *Grewia occidentalis* L.

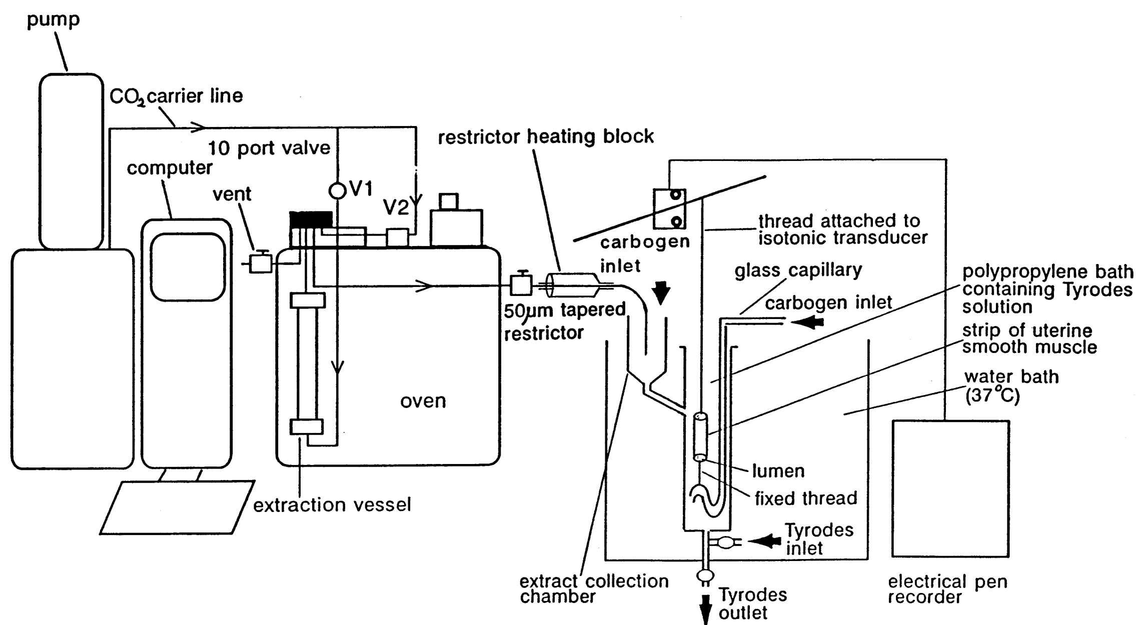

Fig. 1. Schematic diagram of on-line SFE-Bioassay instrumentation.

and *Asclepias fruticosa* L., were evaluated. These plants are used frequently during the late stages of pregnancy. The aqueous extract of *C. miniata* was previously found to be active [20]. Off-line studies have shown the aqueous and SFE extracts of *E. capensis*, *G. occidentalis* and *A. fruticosa* to stimulate uterine contractions in vitro indicating that water modified supercritical CO_2 possessed the solvating ability to extract the uterotonic components. These plants were selected to demonstrate the potential of SFE linked directly to the bioassay, which could be used to rapidly screen various other plants containing uterotonic compounds.

2. Experimental

2.1. Plant material

Fresh plant material was purchased from the Silverglen medicinal plant nursery (KwaZulu-Natal, SA) during October 1996. The wood of *E. capensis*, *G. occidentalis*, and *A. fruticosa* was debarked, finely ground and left to air dry for 72 h. Due to the high moisture content of *C. miniata* the roots were initially dried and then ground into a fine powder.

2.2. On-line SFE-bioassay

The schematic diagram of the on-line SFE-bioassay set up is shown in Fig. 1. SFE was performed using a Lee scientific series 600 SFC pump (Dionex, Sunnyvale, CA) to deliver the SFE fluid, and the series 600 SFC/GC oven to house the extraction vessel. A 24 ml stainless steel extraction vessel (Keystone Scientific; Bellefonte, PA) was housed vertically in the oven maintained at 80°C. A Lee Scientific supercritical fluid extraction injection accessory controlled the passage of CO_2 to and from the extraction vessel. This consisted of a temperature controlled block maintained at the same temperature as the oven and a multiport valve which enabled switching between dynamic and static modes of extraction. As shown by the schematic diagram of the system in Fig. 2, fluid from the pump was initially allowed to pass through on/off valve V1 into the extraction vessel, while keeping the vent valve at position 10 closed,

and the multiport valve in the static mode. This allowed the vessel to pressurise to the required pressure, hence allowing static extraction to proceed for 50 min. The multiport valve was thereafter switched from position 9 to position 8 thereby linking ports 1 and 2 to facilitate dynamic extraction. The 1/16 inch stainless steel tubing connected to port 2 was redirected back into the oven in an attempt to maintain constant temperature during transport of the extracted analytes. Upon exit from the oven, the fluid passed through a 50 μm tapered restrictor (SGE, Australia) and decompressed into the muscle bath at a flow of 18 ml min^{-1} at 150 atm (Fig. 1) SFC grade CO_2 (Air products and chemicals, Allentown, PA) was used as the extraction fluid. Each plant sample was tightly packed into the extraction vessel and 400 μl H_2O added to the matrix. Extraction was carried out at 400 atm and 80°C for 50 min static followed by a 20 min dynamic extraction period. Further, SFE fractions were obtained by sequentially increasing the pressure at constant temperature and modifier concentration. Extractions were performed at 200, 300 and 400 atm, respectively. After the 20 min dynamic extraction period for each sample, the multiport valve was switched back to the static mode (port 9) followed by

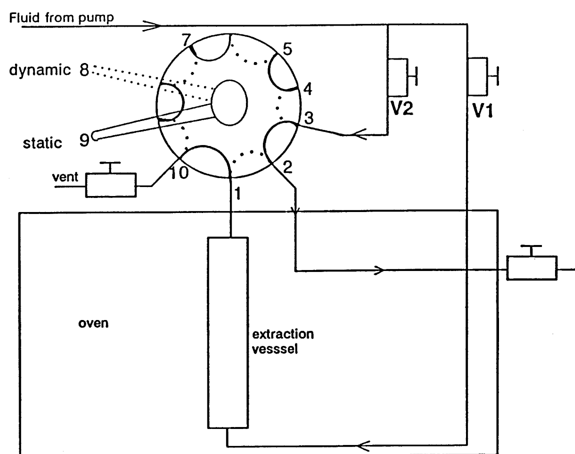

Fig. 2. Plumbing schematic of multiport valve used for switching between static and dynamic modes of extraction.

closure of valve V1 (Fig. 2) Valve V2 was thereafter opened allowing pure CO_2 of the same density as the extracting fluid, to flow through ports 3 and 2. This procedure enabled flushing and cleansing of the 1/16 inch transfer line as well as the tapered restrictor, thereby eliminating the possibility of memory effects during analysis. The vessel was simultaneously vented through port 10.

Mature non-pregnant guinea pigs were obtained from the Biomedical Resource Centre, University of Durban-Westville (KwaZulu-Natal, SA). 20% pentobarbitone (Maybaker, Port Elizabeth, SA) was administered intramuscularly as an anaesthetic, at a dose of 0.2 g kg^{-1}. The uterus was removed by a midline incision into the lower abdominal cavity and washed immediately in Tyrodes solution at 4°C. The bioassay was performed by placing a 2 cm longitudinal strip of uterine muscle into the muscle bath containing 10 ml Tyrodes solution maintained at 37°C. The lower end of the muscle was fixed to a glass capillary tube while the upper end was suspended by a thread attached to a Harvard 386 smooth muscle isotonic transducer (Harvard Apparatus, MA) which transformed the change in muscle length into a proportional electrical signal. A continuous supply of 95% O_2 and 5% CO_2 (MG Fedgas, Durban, SA) was administered through the glass capillary tube at a flow rate of 60 ml min^{-1} to provide tissue oxygenation and act as a suitable buffer. This signal was recorded using an electrically driven Beckmann R511A chart recorder (Beckman, IL) at a chart speed of 0.05 mm s^{-1}. Caution was exercised in ensuring that the lumen of the uterus remained open at both ends during the assay. *o*-Acetylcholine hydrochloride (ACh) (BDH Chemicals, UK) was used as a standard smooth muscle stimulant at a concentration of 1 μg 100 $μl^{-1}$. The extracts were dissolved in 0.9% sodium chloride (Sabax, JHB, RSA) at known concentrations and dispensed into the muscle bath using eppendorf pipets. Physiological fluid (Tyrodes solution) was prepared by dissolving 2.70 mmol KCl, 1.05 mmol $MgCl_2.6H_2O$, 0.40 mmol NaH_2PO_4, 1.80 mmol $CaCl_2$ 137 mmol

NaCl, 11.90 mmol $NaHCO_3$ and 5.60 mmol D-glucose in distilled water. Sensitisation effects from previous extracts were eliminated by using a new strip of muscle for each analysis.

2.3. Muscle bath construction

Muscle baths were constructed in house from 10 ml polypropylene syringes (Terumo, Tokyo, Japan). The first bath was designed for horizontal flow of CO_2 with a tapered restrictor placed directly into the muscle chamber (muscle bath A). Many problems were associated with such a set up, hence a second bath was designed for reasons that are discussed later. This second bath consisted of a second chamber of 6 ml capacity called the extract collection chamber, linked via a detachable side arm to the muscle bath (Fig. 1.) Extraction was performed into the extract collection chamber while the muscle remained in the muscle bath. This chamber functioned as a CO_2 reduction interface thereby eliminating the direct effects of excess CO_2. The muscle bath was filled with Tyrodes solution by upward displacement until the level of Tyrodes in the extract collection chamber had reached a volume of 4 ml.

2.4. pH measurements

The effects of CO_2 on the pH of Tyrodes solution was monitored by using the pH meter 300 (Zeiss, W. Germany) with a combination pH electrode (Beckmann, CA). The instrument was calibrated with pH 7 and 4 buffer solutions supplied by Beckman Instruments (Fullerton, CA).

2.5. Restrictor fabrication

The end of a length of 25 and 50 μm i.d. deactivated capillary tubing (SGE, Australia) was held in the tip of a bunsen burner flame. Tension was applied by hand on both sides of the heated area. As the fused silica melted, the capillary was pulled to a hair like taper and removed from the flame. The restrictor orifice was thereafter adjusted by cutting back the taper and measuring the gaseous flow rate at room temperature and a pressure of 150 atm [21].

3. Results and discussion

3.1. SFE optimisation

Optimisation of conditions in any SFE experiment is important as it ensures maximum recovery of analytes. However, in natural product matrices this is sometimes difficult to achieve unless the structures of the target analytes are known prior to extraction. Since we were dealing with matrices of unknown chemical constituents, conditions were optimised by off-line SFE studies on the basis of total extractable material obtained per unit time as performed in previous studies [22,23]. Although, the optimisation would have been more meaningful if one particular important analyte was targeted, in reality this was not possible at this time because the structural elucidation was not complete and the active components not identified. It was, therefore, uncertain whether the active components followed an extraction profile predicted by the 'hot-ball' model [24] or whether the active components were extracted largely at a particular point within the extraction period.

In the extraction of all three plants, solubility was the controlling factor in achieving an interactive extraction [25]. The extraction pressure could not be raised above 400 atm to increase the fluid density as this was a limitation of the instrument. The extraction temperature could not be lowered since water was used as the modifier and modifiers greatly increase the critical point of the fluid and it was therefore important that the temperature remained high enough to maintain a single phase region during extraction. Another reason for the solubility limitation even after adding a modifier could have been the possible displacement of the modifier out of the extraction vessel upon commencing with dynamic extraction. It was therefore necessary to include an equilibration period during extraction to prevent the modifier from being displaced in order to achieve an interactive extraction. This was accommodated for by using an initial 50 min static extraction period followed by 20 min of dynamic extraction.

Prior to coupling SFE to the bioassay, two factors were considered, viz. the effects of supercritical CO_2 decompression on temperature and pH of the muscle bathing solution.

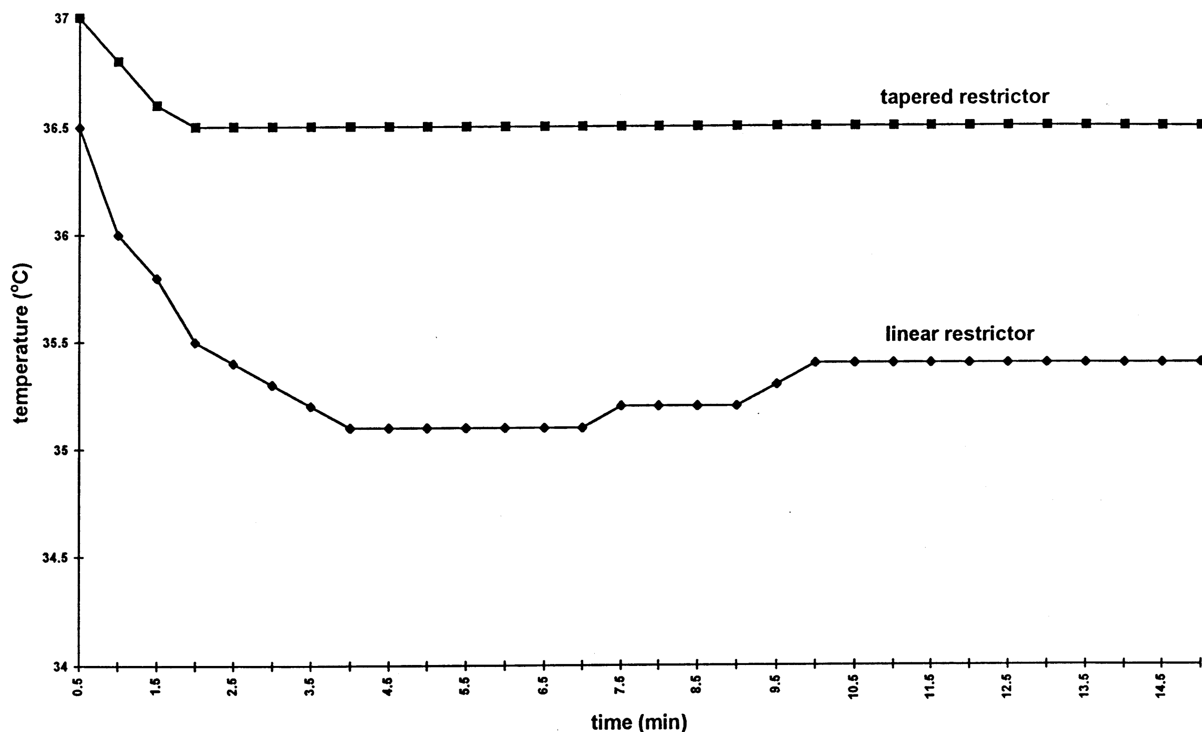

Fig. 3. The effect of CO_2 decompression on temperature of Tyrodes solution. CO_2 was decompressed from an internal pressure of 400 atm through a 25 μm i.d. tapered restrictor (■) and a linear restrictor (◆).

3.2. Temperature effects

Carbon dioxide depressurises at the exit of the restrictor resulting in adiabatic cooling of the collection solvent. One of the problems with this is that the temperature of the collection solvent was not maintained and the collection solvent became so cold that samples containing water caused restrictor plugging from freezing water. Excessive cooling can also alter muscle physiological activity. Veale et al. [20] showed that when the organ bath temperature was reduced to 26°C, spontaneous contractions were inhibited. However, it is unclear whether factors that interfere with spontaneous contractility also affect agonist-induced contractility. There may also be cases where postreceptor mechanisms involved in the response of one agonist are more temperature dependent than those of another [26]. For these reasons, it was decided to study muscle activity at or near body core temperature. Cooling experiments were performed on the first muscle bath with 25 μm i.d. fused silica capillaries. The 24.5 cm × 25 μm i.d. linear restrictor used initially at a pump pressure of 400 atm, reduced the bath temperature to 35.4°C within 10 min (Fig. 3). A tapered restrictor of the same inner diameter as the linear restrictor was subsequently used and found to provide better temperature control and hence, less cooling. The bath temperature decreased to 36.5°C within 1.5 min of commencing CO_2 flow and remained constant at this temperature for the 15 min period of the experiment. Hence, it is quite apparent that the degree of cooling is controlled by the flow of CO_2 or the rate of decompression. Tapered restrictors allow for the flexibility of optimising flow rate by simply cutting back at the taper. By performing such a procedure, the inner diameter of the orifice at the end of the taper increases thereby allowing increased flow rates. Hence a compromise was reached between the rate of decompression and

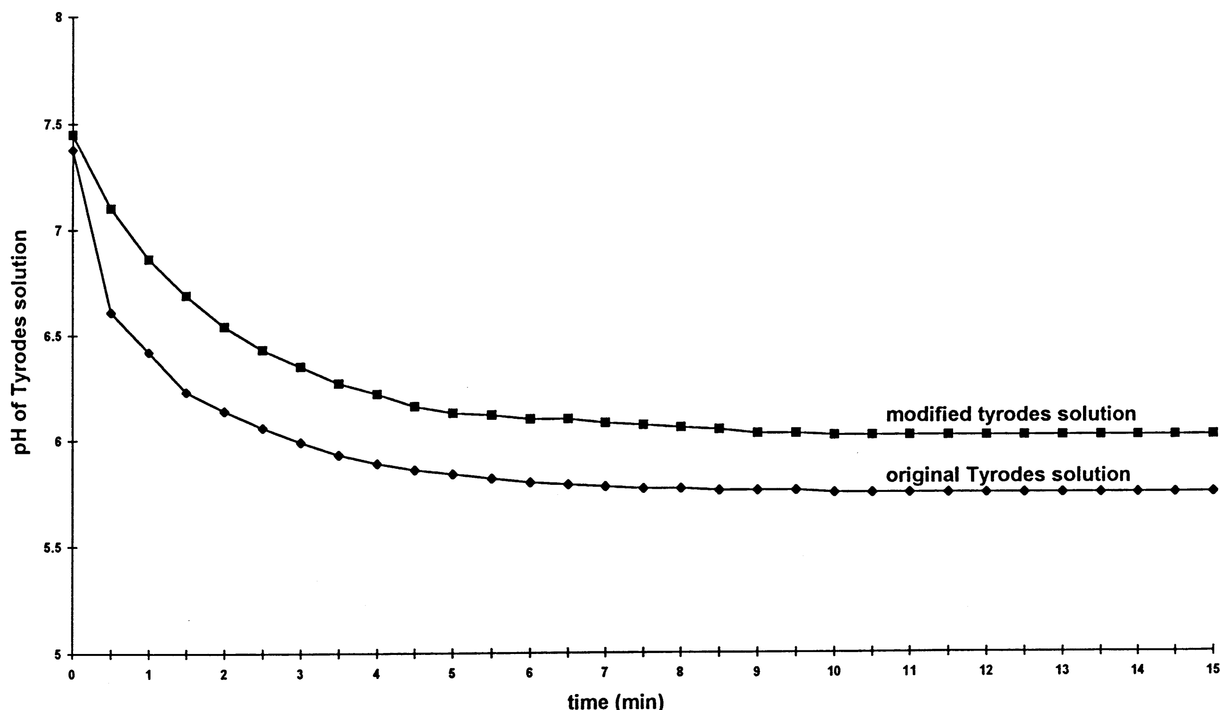

Fig. 4. The effect of CO_2 decompression on pH of modified and original Tyrodes solution. CO_2 was decompressed from an internal pressure of 400 atm through a 25 μm i.d. tapered restrictor. CO_2 flow was measured at 150 atm after decompression and found to be 18 ml min^{-1}.

restrictor plugging, as low flow rates due to small inner diameters of the taper orifice would have led to blockages by the extract. A flow rate of 18 ml min^{-1} at 150 atm was found to be most appropriate and did not result in blockages. The temperature decrease was also minimised and observed to be close to the desired experiment temperature. Despite the problems with cooling, the linear restrictor was further evaluated during extraction of a real sample and was found to plug within 10 min of dynamic extraction. This was due to decompression taking place over the entire length of the capillary resulting in the formation of analyte clusters and small particles during this process. Frit restrictors were also evaluated and were found to provide excellent temperature control due to the low flow rate of CO_2. Frit restrictors are also more resistant to plugging due to their multitude of decompression paths, however the frit failed on many accounts to withstand high pressures.

3.3. pH effects

When CO_2 is introduced into water, it forms carbonic acid resulting in a decrease in pH. Some of the carbonic acid dissociates to bicarbonate and hydrogen ions. Because alteration in $[H^+]$ will alter cellular metabolic processes in which it is a participant, pH measurements were carried out by placing a pH electrode into the muscle bathing solution with CO_2 flow directed into the muscle bath A. As shown in Fig. 4, within 30 s of commencing flow of CO_2, the pH decreased from pH 7.0 to 6.61. The rate of decrease thereafter became moderate and finally remained constant after 10 min at a pH of 5.75. The pH range compatible with life is about 7.8–6.8, however, values as low as 6.0 have been reported for skeletal muscle [27]. A total of 5% CO_2 present in carbogen forms an excellent buffer with bicarbonate present in Tyrodes solution. Since the pK of the HCO_3^-/CO_2 system is 6.1, extracellular fluid

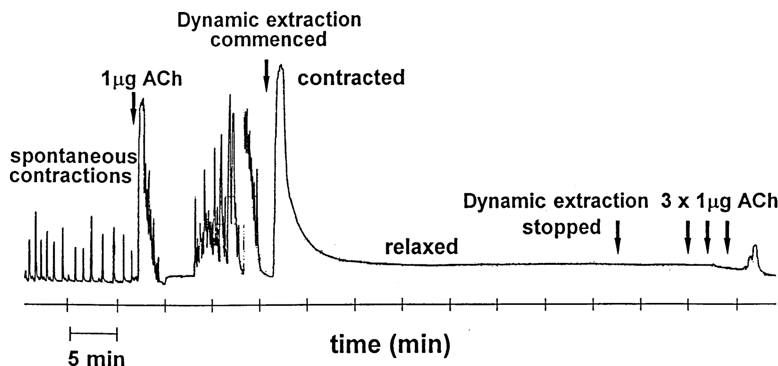

Fig. 5. The direct effect of CO_2 on muscle contractility. Blank extraction was performed at 400 atm and 80°C.

at a pH of 7.4 is not very effective in resisting changes in pH arising from changes in P_{CO_2}. Furthermore, deviations in pH may influence ionization of drugs or charged chemical groups on receptors thereby changing the moieties which interact to produce a response [28,29]. Since CO_2 was used as the extracting fluid in SFE, the P_{CO_2} in extracellular fluid, intracellular fluid and Tyrodes most certainly changed. It was therefore necessary for Tyrodes solution to exhibit buffer action even in the absence of carbogen and this was accomplished by introducing a phosphate buffer as this buffer has a pK value of 6.8. Furthermore, intracellular fluid, with high levels of protein and organic phosphates, are responsible for most of the buffering that occurs when P_{CO_2} changes in biological systems. The concentration of NaH_2PO_4 originally present in Tyrodes solution was reduced from 0.4 to 0.2 mM together with the addition 0.2 mM Na_2HPO_4. Subsequently, the pH over a 15 min period decreased from pH 7.45 to 6.02 and remained constant (Fig. 4). An improvement by 0.27 pH units was observed and this solution was used for all future assays as it provided an improved buffering capacity than the previous solution. Tyrodes solution is a well known nutrient solution capable of preserving tissues in a viable state and although other buffers may have improved buffering capacity, changes in ionic content and composition are known to affect tissue reactivity and base line activity [30,31]. Hence, minimum alterations to the recipe of Tyrodes solution were performed. The physiological activity of a strip of uterine muscle was monitored under these conditions. The muscle was allowed to develop spontaneous contractions and a dose of 1 μg acetylcholine hydrochloride (ACh) was administered as a standard smooth muscle stimulant (Fig. 5). A blank extraction was performed at 400 atm and 80°C and the CO_2 flow directed into the muscle bath via the tapered restrictor. Immediately upon commencing with the dynamic extraction, a contraction was observed. As the extraction proceeded, the muscle began to relax and a flat baseline was thereafter observed. The spontaneous contractions initially observed had ceased altogether. After 45 min, the extraction was stopped and this was followed by the addition of 2×1 μg quantities of ACh. No response was observed, however upon administering 3 μg ACh, a mild response was observed. The effects of CO_2 were further seen when an off-line extract of the wood of *E. capensis* induced muscle contractions. The contractions failed to stop even after administration of mepyramine, a (H_2) histamine receptor blocker. However, when CO_2 was directed from the SFE vessel into the muscle bath, the development of tension was initially noted but this was followed by muscle relaxation and a flat baseline. These results demonstrated that apart from the pH decrease, CO_2 was directly inhibiting spontaneous contractility of the muscle. Upon commencing with the dynamic extraction, the pH of Tyrodes solution decreased resulting in increased tension of the muscle in response to extracellular acidosis.

A similar trend was observed by Cole et al. [32] when investigating contractile responses of isolated human ureteric smooth muscle to extracellular pH changes. However, a continuous influx of CO_2 into the organ bath resulted in the inhibition of muscle activity as CO_2 entered the cell and acidified the intracellular fluid. CO_2 is an easily diffusable gas and is able to pass through membranes and alter intracellular metabolic processes. Alterations in intracellular pH could alter smooth muscle contractility as the following may be pH sensitive: (1) Myosin ATPase activity [33]; (2) the sensitivity of the contractile unit to Ca^{2+} [34,35]; (3) transmitter release and receptor sensitivity [36]; (4) competition at calcium binding sites [37]; and (5) late energy production [38]. Hypoxia could also alter force development and has been shown to impair contractility in tracheal smooth muscle [35]. During hypoxia there is an increase in glycolysis and a concomitant increase in intracellular lactate. Thus, the reduction in force during hypoxia is assumed to be due to a decrease in pH. Nevertheless, no matter what mechanism prevailed in altering the force development during hypercarbia, it was a reversible process. Once the flow of CO_2 ceased and the Tyrodes solution changed, the muscle regained normal physiological activity after passing carbogen for a few minutes. Having undertaken various measures to avoid temperature and pH effects, it was concluded that muscle bath A was not appropriate for on-line coupling since the muscle was in direct contact with the incoming CO_2 from the extraction vessel. Hence, the high P_{CO_2} influenced muscle activity thereby making it impossible to analyse for uterotonic activity induced by plant extracts. Since CO_2 penetrates through cell membranes easily and alters the intracellular activities, this excess had to be minimised. A second muscle bath was therefore designed to eliminate the effects of excess CO_2. It consisted of two polypropylene chambers attached via a detachable sidearm. Dynamic extraction was performed into 4 ml of Tyrodes present in the extract collection chamber while the muscle equillibrated in the 10 ml muscle bath (Fig. 1). In this way the muscle was protected from the direct effects of high P_{CO_2}. The passage of carbogen into the extract collection chamber further assisted in the rapid displacement of CO_2. It was hypothesised that as the dynamic extraction proceeded, the concentration of the extracted material in the extract collection chamber would increase and mix with the Tyrodes solution in the muscle chamber. The carbogen flowing into the muscle chamber facilitated the rapid mixing of the plant extracts until the uterotonic components reached a sufficient concentration to induce a contractile response. If required, the Tyrodes solution from the extract collection chamber could be flushed into the muscle chamber to provide a higher concentration of the extract. A 50 μm i.d. tapered restrictor designed to produce the optimum flow of CO_2 was used in this set up together with the passage of carbogen into the extract collection chamber. The passage of carbogen was found to assist in maintaining the desired pH of the Tyrodes solution by rapidly displacing excess CO_2.

4. Evaluation of extracts

Each plant was initially evaluated by extraction at a pressure of 400 atm and 80°C to ascertain whether the uterotonic components were of sufficient concentration to initiate a muscle response using this new technique. Subsequent analysis was performed by sequentially extracting the components at 200, 300 and 400 atm, respectively, in order to decrease the complexity of the extracts and hence minimise possible antagonistic effects from interfering compounds. Fractionation also aided in determining the most potent extract of the plant which would be useful in further analysis. The response of the muscle to ACh before and after extraction of the plant components was determined for each assay

4.1. Analysis of E. capensis

Following an equilibration period of 30 min, during which time static extraction was performed, the muscle was found to produce an adequate response to 5 μg ACh (Fig. 6a). Ten

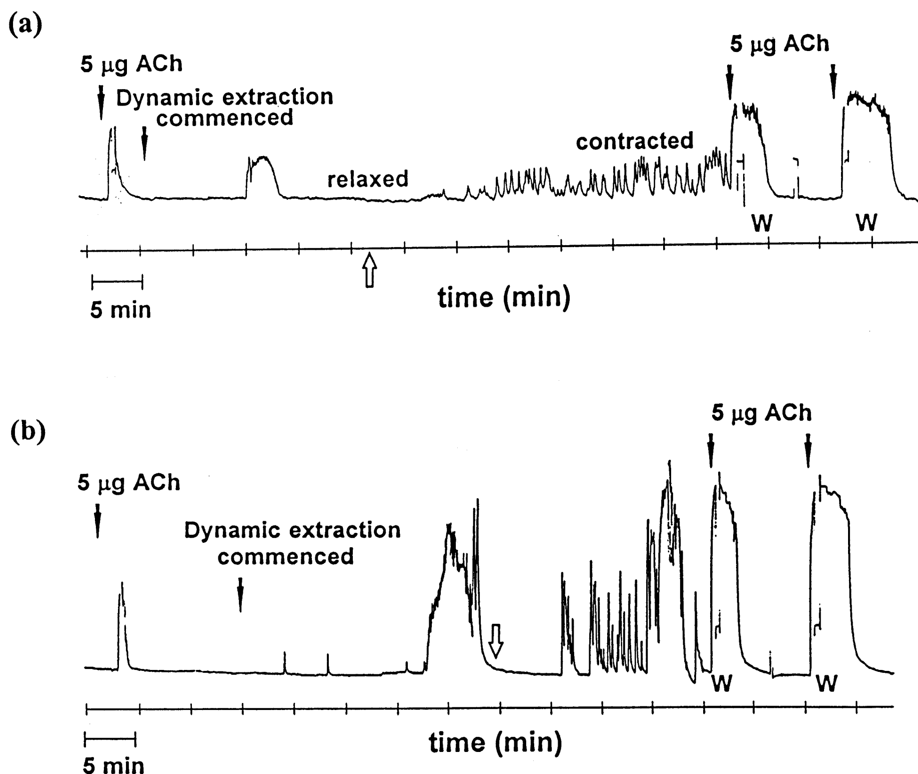

Fig. 6. The effect of SFE extract of *E. capensis* on guinea pig uterine smooth muscle. (a) Total extract obtained at 400 atm, (b) sequentially fractionated extract obtained at 400 atm. ⇑, Point at which contents of the extract collection chamber was transferred to the muscle bath; **W**, muscle wash with Tyrodes solution.

min after dynamic extraction at 400 atm commenced, the muscle was observed to develop tension. The probability of cold zone contracture was ruled out as the temperature during this period was measured and found to be 37°C. The contractile process lasted ~3 min. Once the 20 min dynamic extraction period had lapsed and the contents of the extract collection chamber transferred to the muscle bath, the muscle developed rhythmic activity of increasing amplitude followed by a period of sustained contraction-relaxation cycles, which were observed for a period of 30 min. Subsequent addition of ACh to the muscle bath produced a prolonged response of similar amplitude. A similar cholinergic response following a muscle wash with Tyrodes indicated that the extract may have sensitised the contractile mechanism thereby increasing the agonistic response of ACh. Sequential extractions at 200, 300 and 400 atm were thereafter performed and Fig. 6b displays the contractions induced by the sequentially fractionated 400 atm extract. An increase in muscle tension was observed ~15 min after commencing with the 400 atm extraction. As the extract concentration increased the muscle developed contractile activity of much larger amplitude than the 400 atm total extract indicating that the sequential extract possessed a higher degree of potency due to fractionation. The interfering compounds that may have induced antagonsitic effects may have been removed in the earlier fractions hence leaving behind a purer uterotonic fraction. This extract indeed produced stimulating action followed by an increase in the frequency of contractions. The ACh response was also enhanced even after washing of the muscle with Tyrodes solution.

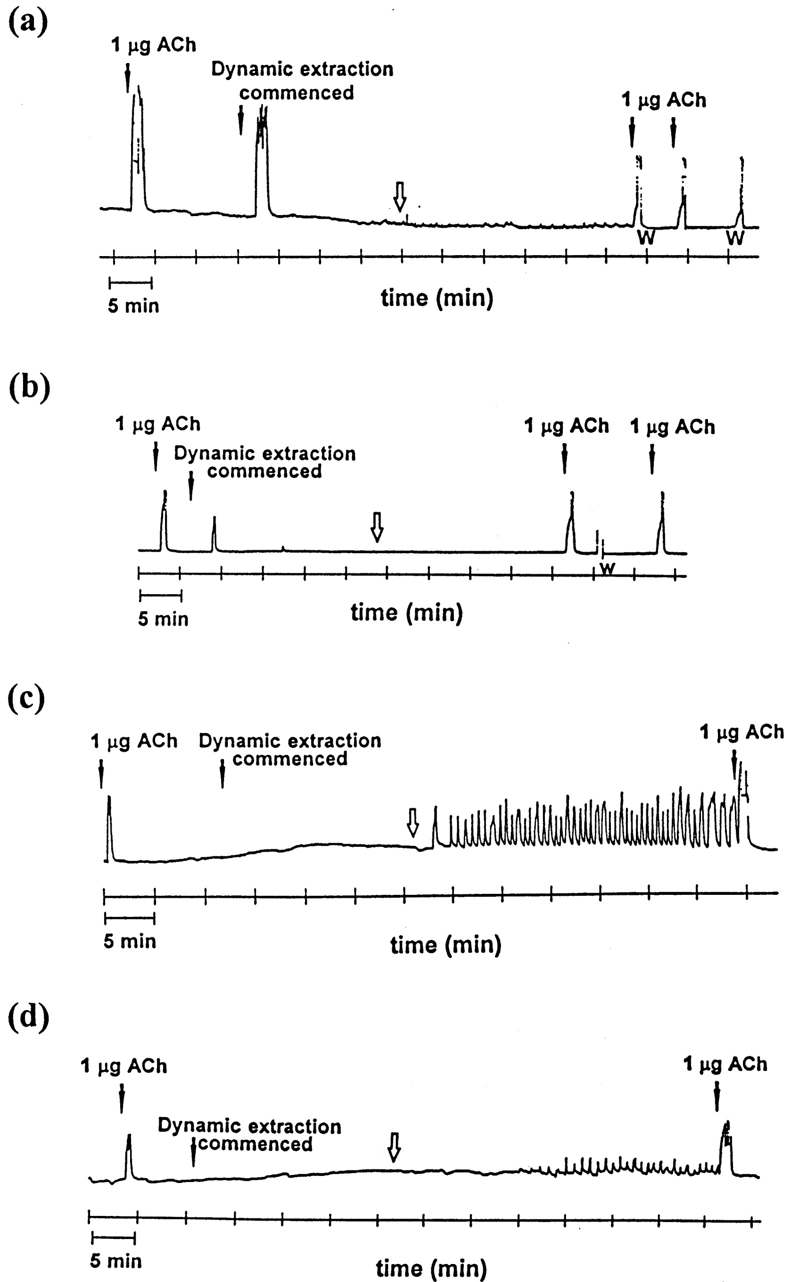

Fig. 7. The effect of SFE extract of *G. occidentalis* on guinea pig uterine smooth muscle. (a) Total extract obtained at 400 atm, (b) sequentially fractionated extract obtained at 200 atm, (c) 300 atm and (d) 400 atm. ⇑ Point at which contents of the extract collection chamber was transferred to the muscle bath; **W**, muscle wash with Tyrodes solution.

4.2. Analysis of G. occidentalis

The total 400 atm extract of *G. occidentalis* produced active tension within 2 min of commencing dynamic extraction, however at the end of the dynamic extraction period, a tonic rhythm of extremely low amplitude was observed. This was followed by an extremely low response to

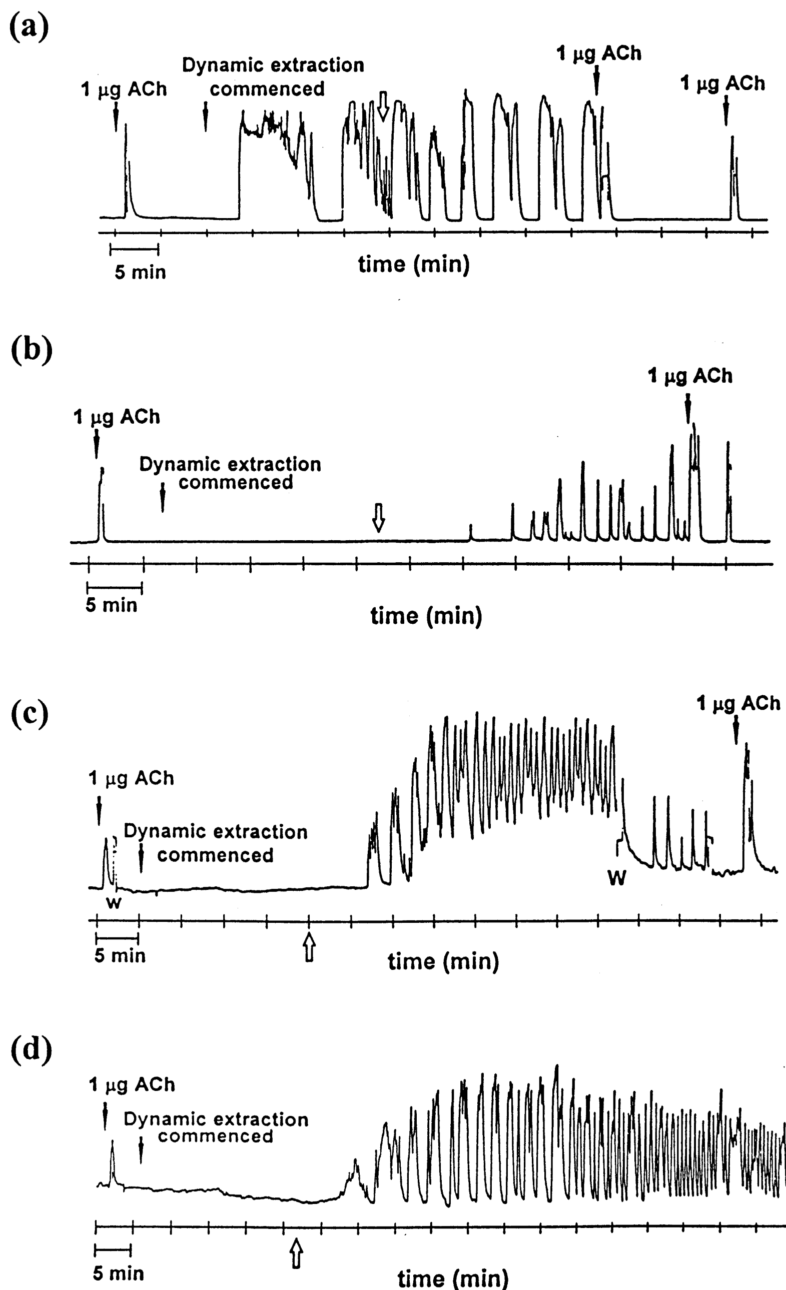

Fig. 8. The effect of SFE extract of *C. miniata* on guinea pig uterine smooth muscle. (a) Total 400 atm extract, (b) sequential extract at 200 atm, (c) 300 atm (d) 400 atm. ⇑ point at which contents of the extract collection chamber was transferred to the muscle bath; **W**, muscle wash with Tyrodes solution.

ACh indicating the possibility of toxic components however this inference remains to be confirmed (Fig. 7a). The 200 atm sequential extract did not provide conclusive evidence of uterotonic activity although a single contraction lasting approximately 30 s was observed (Fig. 7b).

However, the 300 atm extract produced a well synchronised response leading to the production of regular, rhythmic contractions that were monitored for a 30 min period (Fig. 7c). Addition of ACh was observed to produce a contraction similar to the initial response. The 400 atm extract was also found to induce phasic contractions, however, of much lower amplitude than that induced by the 300 atm extract (Fig. 7d). This study clearly demonstrated the potential of supercritical fluid fractionation as the 300 atm sequential extract was observed to be the most potent.

4.3. Analysis of C. miniata

The total 400 atm extract of the roots of *C. miniata* was found to initiate contractile activity within 4 min of commencing with dynamic extraction. An increase in muscle tension was observed together with occasional spiking. The muscle remained in this state of contraction for ~ 8 min, however, upon completion of dynamic extraction an intense contractile activity of high amplitude was detected (Fig. 8a). The contractions were observed to be more irregular, more prolonged and less frequent than the contractions induced by *G. occidentalis*. The 200 atm sequential extract produced regular contractions of varied amplitude (Fig. 8b) while the 300 atm extract was found to develop contractile activity ~ 5 min after stopping dynamic extraction. The contractions were observed to be phasic together with tension development (Fig. 8c). Unlike the activity induced by the 200 atm extract, these contractions were of a greater amplitude and frequency. These contractions were monitored for a 35 min period followed by two muscle washes in order to stop contractile activity. Addition of ACh was found to elicit a much greater response than the initial contraction and this could once again be explained by the sensitizing nature of the extract on the contractile mechanism of the uterine muscle. The 400 atm sequential extract also produced contractions which initially increased in amplitude with periods of quiescence alternating with periods of activity. The contractions were initially observed to be irregular and prolonged however, with time, the amplitude decreased, followed by an increase in the frequency of contractions (Fig. 8d). The decrease in amplitude of the contractions could be attributed to muscle fatigue.

4.4. Analysis of A. fruticosa

This plant, unlike the others, did not require modified supercritical CO_2 to produce uterotonically active fractions. Pure CO_2 was used in the extraction of active components. The total 400 atm extract was found to initiate muscle activity within 7 min of commencing with dynamic extraction. The contractions were of a prolonged, irregular nature of high amplitude. The 200, 300 and 400 atm extracts were all found to stimulate muscle contraction, however, the pattern of muscle activity was found to vary.

5. Conclusions

The results presented herein show that the online assay is a rapid, safe and sensitive method for determining the uterotonic activity of medicinal plants. The selectivity of the extraction was successfully varied through pressure control thereby minimising the possibility of interfering compounds. The method could be adapted to screen plants with other therapeutic potential (e.g. plants used in the treatment of diabetes mellitus and hypertension).

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