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Short communication

Development and validation of a subcritical fluid extraction and high performance liquid chromatography assay for medroxyprogesterone in aquatic products

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ABSTRACT

A simple, rapid and sensitive method was developed for the determination of medroxyprogesterone in aquatic products by extraction with subcritical 1,1,1,2-tetrafluoroethane (R134a) and high performance liquid chromatography (HPLC). A response surface methodology (RSM) was adopted to optimise extraction pressure, temperature and co-solvent volume. The optimum extraction conditions predicted within the experimental ranges were as follows: pressure, 3 MPa; temperature, 25 °C; and co-solvent volume, 6 ml. The analysis was carried out on Zorbax SB-C_{18} column (4.6 mm × 150 mm, 5 μm) with the mobile phase acetonitrile–water (55:45, v/v), flow rate 1.0 ml/min, temperature 30 °C and wavelength 240 nm. Good linearity of detection was obtained for medroxyprogesterone between concentrations of 50–250 ng/ml, $r^2 = 0.999$. The method was validated using samples fortified with medroxyprogesterone at levels of 10, 30 and 50 ng/g, the mean recovery exceeds 90%, and the RSD values were less than 10%.

1. Introduction

Medroxyprogesterone has been used extensively in stockbreeding and can significantly improve the economic efficiency of husbandry. However, medroxyprogesterone residue in animals used for food could endanger the health of consumers. In many countries, the use of medroxyprogesterone is prohibited in food-producing animals. Analytical methods based on liquid–liquid extraction, solid-phase extraction, high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC–MS) are available to control the illegal use of medroxyprogesterone in food-producing livestock [1–4]. These methods, however, are often time- and organic solvent-consuming.

Supercritical fluid extraction (SFE) is a potentially attractive alternative to conventional methods for the recovery of anabolic steroids [5,6]. This technique offers shorter extraction times with high recoveries and low consumption of organic solvents. Supercritical carbon dioxide is the most popular solvent for supercritical fluid extractions. However, in view of economic and environmental issues, alternative SFE solvents that operate under less severe conditions should be explored, as CO_2 typically requires a pressure of up to 300 bar for satisfactory extraction.

This drawback can be eliminated using common 1,1,1,2-tetrafluoroethane (R134a) instead of CO_2. Because R134a is non-flammable, has low toxicity, and is not an ozone-depleting substance, it has been considered to be a potential replacement for the refrigerant Freon-12 (R12). In addition to its commercial availability, the permanent dipole moment (2.05 D) and reasonable critical properties (101.1 °C, 4.06 MPa) of R134a have led to the evaluation of its use as an alternative to supercritical CO_2 for the extraction of polar compounds [7]. In the high-pressure and low-compressibility region, the solvating power of R134a is greater than that of CO_2 [8]. High extraction efficiency could be achieved at low pressure with subcritical fluid extraction technology, which overcomes the traditional shortcomings of SFE.

The scope of the current work covers the evaluation of R134a for the subcritical extraction of medroxyprogesterone from aquatic products. Subcritical extracts were quantified and validated using high performance liquid chromatography (HPLC) for the determination of medroxyprogesterone in samples. The extraction pressure, the temperature and the volume of co-solvent were investigated and optimised to achieve an efficient extraction. The method was evaluated by analysing medroxyprogesterone in a real tilapia sample, which was fed a diet containing medroxyprogesterone.

2. Material and methods

2.1. Chemicals and reagents

The extraction solvent was supercritical fluid-grade R134a (INEOS, England). HPLC-grade methanol (Merck, Darmstadt,

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Germany) was used throughout the experiment. Medroxyprogesterone was purchased from Dr. Ehrenstorfer (Germany). Solid phase extraction (SPE) cartridges (C_{18}, 6 cm^3) were obtained from the Waters Corporation (Milford, MA, USA).

2.2. Animal tissue

Tilapia fish were filleted, their skin and bones were removed, and their muscles were blended in a laboratory blender. The tissue samples were frozen at −20 °C prior to analysis.

2.3. Apparatus

The instrument used in the subcritical R134a extraction experiments was made at home. It consists of a high-pressure pump (Hangzhou Zhijiang Petrochemical Equipment Co., Ltd., Hangzhou, China) capable of generating a maximum pressure of 350 bar and a maximum flow rate around 0.4 l/h. The instrument has an oven and equipped with a extraction vessel (120 mm × 20 mm I.D.) and a separator (120 mm × 20 mm I.D.) that can be operated at pressure up to 300 bar. Liquid R134a is handled by a high pressure metering pump with jacketed head for cooling, and the flow rate could be regulated between 0.0 and 0.4 l/h.

2.4. Subcritical R134a extraction

One kilogram of tilapia muscle tissue was spiked with a final concentration of 3 μg/g medroxyprogesterone for use in the evaluation of the subcritical extraction. Optimisation experiments were carried out on a 5-g sample of tilapia tissue. The tilapia tissues were mixed with 15-g quartz sand and 5-g fibreglass to disperse the sample fully. In each experiment, the sample was placed in the middle of an extraction cell, and co-solvent was added. The bottom and top of the extraction cell were filled with glass wool to prevent entrainment of the material and blockages in the system.

The extraction cell was placed in a heating bath to maintain an operating temperature within ±1 °C of the set-point temperature for each run. R134a was delivered via a metering pump with a flow rate of 10 g/min. The system pressure was controlled by a back-pressure regulator that was adjusted to maintain pressure in the range of 20–150 bar. To improve the extraction efficiency, a static period of 20 min was allotted to promote contact between the sample and the subcritical R134a fluid. The static period was subsequently followed by dynamic extraction for 40 min. Next, the stream of subcritical R134a fluid containing medroxyprogesterone was depressurised through a pressure restrictor, and the extracts were collected in a separator with 10 ml methanol.

2.5. SPE clean-up

To decrease interferences and increase sensitivity, the acquisition of clean extracts is highly desirable, especially for the analysis of biological samples. The methanol solution containing the extracts was evaporated to dryness under reduced pressure at 40 °C, diluted with 2.0 ml of a methanol:water (1:1) solution, and applied to a preconditioned solid-phase extraction (SPE) cartridge (C_{18}, 6 cm^3, preconditioned with methanol (2.0 ml) and water (2.0 ml)). After washing with 3 ml of water, medroxyprogesterone was eluted with 5 ml of methanol. The eluent was evaporated to dryness, diluted to volume with the mobile phase, and quantified by HPLC.

2.6. Chromatographic conditions

An Agilent 1100 series LC system was used in this study. The separation was conducted on an Zorbax SB-C_{18} column (5 μm, 4.6 mm × 150 mm) equipped with a guard column. The column temperature was maintained at 30 °C. The mobile phase, consisting of acetonitrile:water (55:45, v/v), was pumped at 1.0 ml/min. Medroxyprogesterone was detected at 240 nm (bandwidth, 4 nm) for quantification using a diode array detector (DAD).

2.7. Method validation

Tilapia tissue samples fortified at different levels (5–50 ng/g) were extracted, and linearity and sensitivity were checked by injecting the medroxyprogesterone extracted into the HPLC. The limit of detection (LOD) was based on the 3 s criterion. Finally, the limit of quantification (LOQ) was selected as the lowest concentration used in the calibration curve.

In addition to LOD and LOQ, accuracy and precision were studied. The fish samples that were fortified with medroxyprogesterone at levels of 10, 30 and 50 ng/g were extracted and analysed on 3 different days. Each group repeated 4 times. The muscle tissue of the tilapias that were fed a diet containing medroxyprogesterone was extracted using subcritical R134a to evaluate this method.

2.8. Box–Behnken design

Many factors can affect the efficiency of a subcritical R134a extraction, including pressure, temperature, co-solvent volume, flow rate, extraction mode and extraction time. Therefore, multiple variables may influence the extraction efficiency. The response surface methodology (RSM) is an effective technique for optimising the process [9]. According to preliminary experimental results, the significant variables, such as pressure, temperature and co-solvent volume, were selected as the critical variables and designated X_1, X_2 and X_3, respectively. The low, middle, and high levels of each variable were designated as −1, 0, and +1, respectively.

Polynomial regression equations were developed to describe the effects of the 3 independent processing parameters—extraction pressure (X_1, MPa), extraction temperature (X_2, °C) and co-solvent volume (X_3, ml)—on the extraction rate of medroxyprogesterone. The independent variables—extraction pressure (X_1) (3, 9 and 15 MPa), temperature (X_2) (25, 40, and 55 °C), and co-solvent volume (X_3) (2, 4, and 6 ml)—were varied to investigate their effects on the extraction rate of medroxyprogesterone. The general form of the quadratic polynomial model regression equation employed in this study is presented in Eq. (1), as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

in which Y is the predicted response (extraction rate %); β_0 is a constant; β_i, β_{ii} and β_{ij} are the linear, quadratic and interactive coefficients, respectively, and X_i and X_j are the levels of the independent variables.

Three-dimensional surface response plots were generated using the fitted model by altering two of the variables within the experimental range while holding the other constant at the central point. The Design Expert software package was used for regression analysis and analysis of variance (ANOVA). The test of statistical significance was based on the total error criteria with a confidence level of 95.0%.

3. Results and discussion

3.1. Fitting the models

The medroxyprogesterone extraction rates from each of the experiments are listed in Table 1. The experimental data were analysed by analysis of variance (ANOVA), and the results are listed

Table 1
Experimental points of the Box–Behnken design and the experimental data.

Experiment number	X_1	X_2	X_3	Extraction rate (%)	
				Experimental	Predicted
1	−1	−1	0	82.91	84.66
2	1	−1	0	85.93	86.71
3	−1	1	0	92.72	91.93
4	1	1	0	89.23	87.48
5	−1	0	−1	57.67	55.27
6	1	0	−1	72.67	71.23
7	−1	0	1	93.19	94.63
8	1	0	1	73.85	76.26
9	0	−1	−1	54.30	54.95
10	0	1	−1	58.53	74.12
11	0	−1	1	95.49	92.30
12	0	1	1	81.81	81.16
13	0	0	0	85.73	87.15
14	0	0	0	86.31	87.15
15	0	0	0	89.41	87.15

in Table 2. The model with a p-value less than 0.01 was statistically significant and was therefore suitable for this experiment. The "lack of fit" of this model was insignificant with a p-value of 0.217. The coefficient of determination (R^2) and adjusted coefficient of determination (Adj. R^2) were 0.9762 and 0.9334, respectively, indicating adequate accuracy and general availability of the polynomial model.

ANOVA was also used to evaluate the significance of the coefficients of the models. For any term in the model, a large regression coefficient and a small p-value indicate a more significant effect of that term on the respective response variables [10]. Thus, the variable with the largest effect on the extraction rate was the linear term of co-solvent volume ($p < 0.001$) followed by the quadratic terms of co-solvent volume ($p < 0.01$). The interactions between the pressure and co-solvent volume and the interactions between the temperature and co-solvent volume had significant effects on the extraction rate.

3.2. Analysis of response surfaces

Surface response plots of the model can allow one to visualise the effect of the independent variables on the dependent variables, and they were created by adjusting two variables within the experimental range and holding the other variable constant at the central point (Fig. 1) [11].

The results show that the extraction rate increased with pressure at low temperature levels. This observation can be explained using the established principles of SFE technology for gases and fluids [12–15]. Increasing the pressure around a subcritical state causes the density of the R134a solvent to increase, and the intermolecular interactions of the solutes increase. As a result, the dissolution of the medroxyprogesterone was promoted, thereby increasing the amount of medroxyprogesterone extracted. At high pressure levels, however, the extraction rate decreased, perhaps because the state of the R134a fluid was far from the subcritical state. This change of state could affect the extraction ability of R134a. The viscosity also increases with pressure, which could prevent the solute from spreading to the fluid. Not that the co-solvent was added to the extraction cell prior to the dynamic extraction. A high extraction pressure may result in a low polarity of the subcritical fluid because high-density R134a fluid (which swells with increasing pressure) would decrease the percentage of the co-solvent. Therefore, the extraction rate decreased with increasing pressure.

The co-solvent volume had a positive linear effect on the extraction rate at low levels, which is probably due to the improvement of R134a polarity with the increase in co-solvent volume. For large co-solvent volumes, however, the negative effect became significant, as reflected in the plateau of the extraction rate for the co-solvent volume over 5 ml. Note that methanol can enhance the polarity of subcritical R134a fluid and is therefore the co-solvent favoured for the dissolution of medroxyprogesterone. It should also be noted, however, that large amounts of co-solvent will change the critical parameters of the mixtures. Thus, the dissolving capacity of subcritical R134a fluid decreased.

Both the extraction temperature and the co-solvent volume significantly affected the medroxyprogesterone extraction rate. Similar effects were observed with changes in the pressure and co-solvent volume. The impact of the temperature on the extraction rate was found to be more dramatic at low co-solvent volumes. Conversely, this impact was small at high co-solvent volumes. As expected, the mass transfer rate was faster at higher temperatures [16]. The vapour pressure of the solutes (which rises with increasing temperature) at higher temperatures also play a role in increasing the extraction rate. When the co-solvent volume was too low, the solubility of the medroxyprogesterone largely depended on solute vapour pressure. Note that the higher the temperature was, the quicker the volatilization of co-solvent, and the density of the R134a solvent decreases. Therefore, a higher extraction rate was obtained at low temperatures when the co-solvent volume was large.

Table 2
Estimated coefficients of the second order response model.

Regression coefficient	Extraction rate (Y)		Probability (p)
	Regression coefficient	Standard error	
β_0	87.15	1.864	
Linear			
β_1	−0.6012	1.142	0.621
β_2	−2.0075	1.142	0.139
β_3	11.0963	1.142	0.000
Quadratic			
β_{11}	−0.3700	1.680	0.834
β_{22}	0.9157	1.680	0.609
β_{33}	−12.4350	1.680	0.001
Interaction			
β_{12}	−1.6275	1.615	0.360
β_{13}	−8.5850	1.615	0.003
β_{23}	−7.5775	1.615	0.005
R^2	0.9762		
Adj. R^2	0.9334		

3.3. Optimisation of the extraction conditions

The subcritical R134a extraction conditions were considered optimised if the extraction rate reached its maximum value. From the solutions predicted by the model, experimental conditions under which the pressure was set at 3 MPa, the temperature at 25 °C, and the co-solvent volume at 6 ml could yield an extraction rate of 99.49%. Due to errors inherent in the model and the test measurements, the experiments were conducted under optimised conditions and yielded an extraction rate of 96.60%, which was similar to that predicted by the model.

3.4. Results of the method validation

The method was validated using the developed conditions (temperature, 25 °C; pressure, 3 MPa; co-solvent volume, 6 ml). The calibration curves were linear ($r^2 = 0.999$) over the range of 50–250 ng/ml. The limit of quantification for the method, as determined from the lowest standard on the calibration curve (50 ng/ml), was 10 ng/g (S/N = 3). The accuracy and precision of the method were determined using fish samples fortified with medroxyprogesterone at levels of 10, 30 and 50 ng/g. The mean recovery of

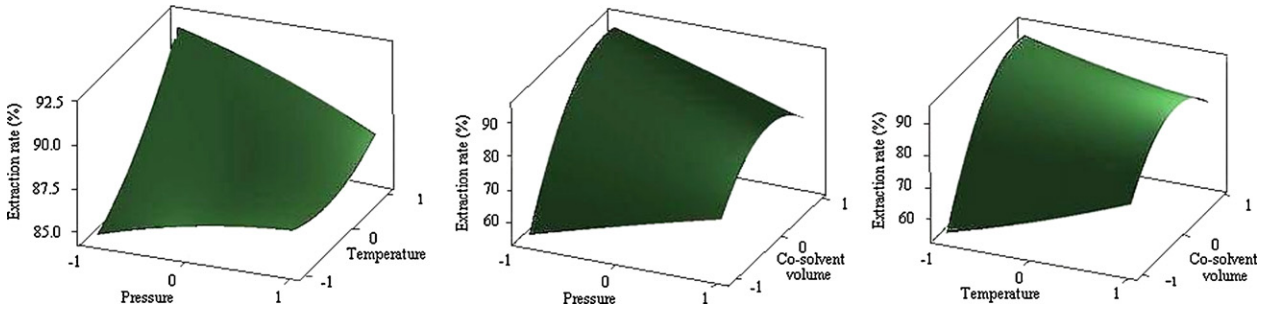

Fig. 1. Surface plots of the medroxyprogesterone extraction rate.

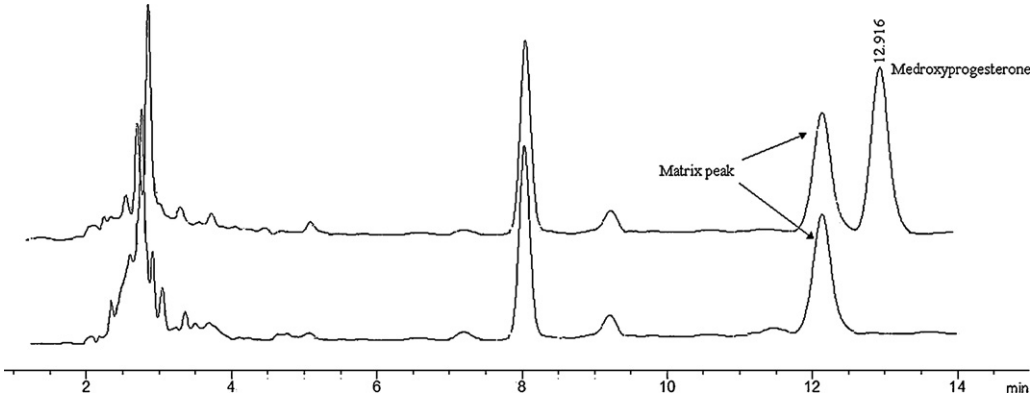

Fig. 2. Chromatograms of medroxyprogesterone and negative control.

Table 3
Extraction recoveries of medroxyprogesterone from tilapia.

Analyte	Fortification level (ng/g)	Mean recovery (%)	RSD (%, $n=4$)
Medroxyprogesterone	10	96.91	5.43
	30	94.33	2.61
	50	94.87	3.20

medroxyprogesterone was above 90%, and the RSD values were below 10% (Table 3). Fig. 2 shows chromatograms for fortified tilapia samples extracted using subcritical R134a.

3.5. Analysis of real samples

The validity of this method was studied using real tilapia samples. Initially, the tilapias were fed a diet containing 50 g/kg of medroxyprogesterone twice a day. After an acclimation period of 30 days, the tilapias were taken from the aquarium for analysis. The results indicate that the tilapia tissues contained 26.3 μg/kg of medroxyprogesterone, which suggests that this is a valid method for detecting medroxyprogesterone in aquatic products.

4. Conclusion

A subcritical extraction method has been developed for the analysis of medroxyprogesterone in aquatic products. This method yields acceptable recovery and repeatability. Under the optimised conditions, the recovery of medroxyprogesterone from tilapia exceeds 90%, and the RSD is less than 10%, proving that the subcritical R134a extraction is a feasible sample pretreatment technology for the analysis of medroxyprogesterone.

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