Methods

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PAPER

Development of a novel analytical approach combining the quantification of amino acids, organic acids and glucose using HPLC-UV-Vis and HPLC-MS with screening *via* NMR

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A simple, rapid, sensitive and selective procedure based on the combination of HPLC-UV-Vis and HPLC-MS has been developed and single laboratory partially validated for the determination of a set of 13 analytes present in a commercially available IVF medium utilising small sample volumes (20–30 μL). The composition fingerprint of the complex sample obtained by NMR spectroscopy in 11 minutes provided identification based on a screening of the metabolomic profile. HPLC-MS allowed the glucose–sodium adduct to be measured accurately and the working and linear ranges achieved were 0.028–0.389 mmol L^{-1} with a detection limit of 13 μM. HPLC-UV-Vis allowed accurate concentrations of pyruvic and lactic acids with linear ranges over 0.005–0.1 mmol L^{-1} with a limit of detection of 28 μM for pyruvic acid to be determined in 8 minutes, while lactic acid presented a linear range over 0.1–2 mmol L^{-1} with a limit of detection of 1.2 mM possible. The use of HPLC-UV-Vis allowed the chromatographic separation of 8 amino acids (aspartate, glutamate, serine, glycine, asparagine, glutamine, alanine, and proline), the dipeptide alanyl-glutamine and taurine previous to a chemical derivatization, providing a total run time of 40 minutes. The method was partially validated to show a linear range of 0.028–0.280 mmol L^{-1} with detection limits ranging between 1 and 30 μM. Development of the analytical approach provided determination and quantification of a set of 13 analytes from a very complex sample. Although well established analytical techniques were used here, combinatory methodologies were partially validated for the first time to this purpose. The novelty of the combination of techniques relies on a screening tool and a strategy to the future evaluation and an improved assessment of human embryo viability.

1. Introduction

Requirements in reproductive biomedicine for the improvement of *in vitro* fertilization (IVF) rates involve the evaluation of embryo morphology, the embryo viability to implant, and the optimum complex medium composition.[1] Parameters evaluated to select the best embryo to transfer are embryo morphology and embryo development during cleavage from blastocysts to morula stage.[2] However, contrary to the expected results, sometimes embryos considered as inappropriate may end up with a positive development while conversely embryos considered as appropriate may not.

Immense effort has been put into the study and optimisation of the perfect culture medium composition because although the embryo nutritional needs in early stages of development are reflected in the female tract, *in vitro* conditions are not definitely known and consequently the necessary elements for the cell growth are unknown.[3] For example, during the first day of cleavage *in vitro*, the embryo has a low metabolic rate, with a limited capacity to use glucose and so generates energy from low level oxidation of pyruvate, lactate[4] and nonessential amino acids.[5,6] After the compaction, the embryo begins consuming glucose[4] through oxidative glycolysis and utilises nonessential and essential amino acids for cellular proliferation and differentiation.[2,3,7,8] Generally amino acids are critically involved in functional pathways including the biosynthesis of intermediates, metal chelators or antioxidants, the sources of energy and also are related with the regulation of the energy metabolism.[9] Several strategies have been proposed to improve the embryo culture medium,[3,11] and recent research in the field has focused upon

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examining correlations between the consumption and excretion of metabolites with success of implantation.[10] Since the 1990s some researchers have attempted to monitor metabolites such as lactate and pyruvate as reported by Conaghan and co-workers,[11] and others have focussed on glucose[9,12] and their effects on the determination of pregnancy rates in embryos.

Most of these reported analytical determinations entail complex and lengthy analysis requiring expensive equipment such as ultramicrofluorescence spectroscopy.[13] This analytical approach has been reported to be useful for the determination of the above metabolites using small sample volumes, typically microlitre volumes of a simple culture medium which lacks essential components such as amino acids. However, the scenario is completely different when analysts wish to tackle the analytical monitoring of a wide number of metabolites, including amino acids, vitamins, and proteins.

More recently, despite the complexity of commercial culture media, non-invasive and elaborate methodologies used in metabolomics and based on chemical monitoring,[14] proteomic assessment,[15] and spectroscopic techniques[10,16–19] have emerged and they have been used, in addition to the morphologic methods or genetic methods,[20] to assess embryo viability. These studies have facilitated and improved the effectiveness of assisted reproduction techniques (ARTs), enabling better embryo selection and reducing the number of transferred embryos.

In that respect, metabolomics is the science of small molecules which are involved in biochemical processes and it has been shown that this approach can provide a great deal of information regarding the "real-time" situation of a biological system within a biochemical approach.[21] Metabolomics is the process of simultaneous monitoring and evaluating changes and dynamics of biological systems.[22] The need of analysing simultaneously the plethora of metabolites in biological fluids such as cell or tissue extracts, urine, plasma, and seminal, amniotic[23] or cerebrospinal fluids has consequently made metabolomics an encouraging science which can provide an integrated view of the whole system.[24] Note that not only is metabolomics focused on biomedical studies but also has uses in other areas such as studying wine fermentation and evaluating malolactic fermentative characteristics of yeast strains.[25,26] The majority of these metabolomic studies include HPLC chromatography coupled with UV-Vis, fluorescence, refraction index or electrochemical detectors, as well as more sophisticated techniques such as mass spectrometry and high field nuclear magnetic resonance. Particularly, some of these have been used for the monitoring of embryonic culture media, including the study of amino acids (alanine, glutamate and leucine) and glucose, lactate and pyruvate[17] *via* NMR and the analysis of embryo protein expression (secretome) by surface enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF-MS) by using ubiquitin as the protein biomarker.[27] We note that the current state of the literature reflects individual techniques towards determining these identified metabolites and it appears that a complete combinatory approach has not been reported towards metabolomic studies, particularly in IVF culture media.

Consequently, in this paper, we report a combinatory pool of the main analytical techniques employed for metabolomic studies which are based on conventional liquid chromatography (LC), mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR).[28] Mass spectrometry requires a pre-separation of the metabolic components using either LC or gas chromatography (GC). To the best of our knowledge, there are no reported studies on the characterization of a commercial embryo culture medium using this combination of techniques. Additionally, in this work, we exploit LC as a routine and accessible technique at many chemical and biological laboratories for the determination of ten amino acids previously *via* derivatisation as well as glucose and two organic acids, lactic and pyruvic. Simultaneously, we take advantage of mass spectrometry and NMR as complementary techniques to LC. In that respect, due to the complexity of isolation of interferences such as hyaluronic acid (HA, an anionic, non-sulfated glycosaminoglycan) present in the culture media, glucose required the use of LC coupled with mass spectrometry. Furthermore, NMR spectroscopy is a powerful, interdisciplinary method and a non-destructive technique which can provide information on the molecular structure of both pure compounds and complex mixtures as well as information regarding absolute or relative concentrations. In particular, the NMR results obtained here, using 800 MHz for the first time, contribute simultaneously to both the determination of new molecules and act as a general fingerprint of the whole metabolomic profile of the culture medium.

The aim of this study is to combine well-known analytical techniques such as HPLC-UV-Vis, HPLC-MS and a more powerful technique such as high field ^{1}H (800 MHz) NMR spectroscopy to separate and determine a complex commercially available IVF culture medium. Moreover, partial validation of our method enhancement has been carried out taking into consideration the following issues: sensitivity, selectivity, limit of detection, limit of quantification, linearity and range. The IVF medium constitutes among others glucose, organic acids: lactic and pyruvic, amino acids: aspartate (Asp), glutamate (Glu), serine (Ser), glycine (Gly), asparagine (Asn), glutamine (Gln), alanine (Ala), and proline (Pro), the dipeptide alanyl-glutamine (Ala-Gln) and taurine (Tau), and other compounds such as hyaluronic acid (HA) and proteins like human serum albumin (HSA). Herein we present a combinatorial approach for the overall determination of analytes in this complex medium by sensitive, rapid, simple and selective techniques which allow the exclusion of interferences. The major achievement of this work for the embryological community will be supported on the basis of the determination of a wide number of analytes. Such results could be used for the evaluation and further comparison of the real culture medium after the embryo incubation process and therefore to the optimised embryo selection yielding in an improved implantation.

2. Materials and methods

Chemicals used were of analytical grade which include D_2O (Sigma, 99.9%), sodium 3-(trimethylsilyl)propionic acid (TSP) (Sigma, 99.9%), formic acid, acetic acid and *ortho*-phosphoric acid (Sigma, 99.9%), phenylisothiocyanate (PITC, Acros Organics, 99%), ethanol (Merck, absolute analysis) and acetonitrile (Scharlau, HPLC grade). Sodium pyruvate, sodium lactate, glucose, Asp, Glu, Gly, Asn and Pro were purchased from Fluka with a purity >99.9%. Ala, Ser, Gln and the Ala-Gln

were purchased from Merck, with a purity >99%. Buffer salt solutions consisted of pH 5.5 sodium acetate adjusted with acetic acid from Scharlau and pH 7.0 potassium phosphates from Fluka. All solutions were prepared with doubly deionised water of resistivity not less than 18.2 MΩ cm^{-1}.

The IVF culture medium (G-1™ v5 PLUS) was purchased from Vitrolife (Göteborg, Sweden), and was used as received and also contained other analytes: Ala, Ala-Gln, Asn, Asp, Glu, Gly, Pro, Ser and Tau; glucose, sodium lactate, sodium pyruvate, sodium citrate, lipoic acid, gentamicin, HA and HSA. The pH for this buffered medium measured at 37 °C and an atmosphere of 6% CO_2 was 7.29 and the osmolality was 264 mOsm kg^{-1}. The supplying company wishes to keep the quantitative composition of this medium confidential and no concentrations have been reported in the literature. Therefore, the composition of this medium is only qualitatively known. The medium was stored at −20 °C and therefore it was thawed at room temperature for 15 minutes prior to vortexing for 6 seconds. Samples were filtered through a 0.45 μm PTFE filter prior to analysis. The experimental procedure of frosting and defrosting the samples from −20 °C up to room temperature was found to have no effect on the different cycles as low variability in the results was shown.

NMR experiments were performed with a Bruker Digital Advanced 800 MHz (Bruker, Germany) coupled with a cryoprobe and settled at 300 K. A portion of 20 μL of samples was used because this is the expected volume in real samples. In order to minimize the dilution of the samples, 3 mm NMR tubes (Norell, Landisville, USA) were used. So, 20 μL of samples were diluted to 150 μL in 0.9% NaCl, 1 mM TSP as internal reference and 10% D_2O. To analyze low molecular weight metabolites, a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence[29] was acquired with 128 scans, a spectral width of 20 ppm and 65k data points, and the experimental time was *ca.* 9 minutes. A 5 s relaxation delay was applied between scans. For further analysis of metabolites 1D-nuclear Overhauser effect spectroscopy (NOESY) spectra were used with 10 ms of mixing time, 128 scans and a relaxation delay of 5 s. For the assignment of the metabolites, *J*-resolved ^{1}H and 2D- ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) experiments were used. Spectra were Fourier transformed and adjusted with TopSpin 2.1 (Bruker BioSpin, Rheinstetten, Germany). Spectra were calibrated using TSP as a reference, and metabolites were assigned using Amix software in combination with the Bruker NMR Metabolic Profiling and BioMagResBank.

Glucose analysis was carried out with HPLC coupled with a mass spectrometer (Agilent 1100 LC/MSD Trap SL model) with a positive ion polarity in the electro spray ionisation (ESI).[30] The column used was a LichroCART, with LichroSpher as the stationary phase, 4.0 × 250 mm, 5 μm, (Agilent Technologies). The mobile phase consisted of a mixture of acetonitrile/water 75 : 25 v/v + 0.1% formic acid, and an isocratic flow rate of 0.5 mL min^{-1}. The injection volume after dilution up to ten-fold with water was 10 μL. Positive ionization was chosen for glucose determination and in all cases before glucose determination, calibration curves were obtained following the ion abundance of 203-mass corresponding to the mass of the adduct [Glucose + $Na]^{+}$. Calibration curves for determination of glucose were performed over days in order to follow the possible variations of the equipment associated with electrospray conditions, type of source or sample contamination.

Table 1 HPLC gradient for amino acids determination

Linear gradient composition[a]		
Time/min	*A* (%)	*B* (%)
0.0	100.0	0.0
21.0	96.5	3.5
29.0	96.5	3.5
40.5	92.5	7.5
42.5	92.5	7.5
45.5	0.0	100.0
65.5	0.0	100.0
76.0	100.0	0.0

[a] Solvent composition *A*: 50 mmol L^{-1} sodium acetate adjusted to pH 5.5 with acetic acid, *B*: acetonitrile.

Lactic and pyruvic acids concentrations were determined by HPLC (Agilent 1100 series, Santa Clara, USA) coupled with an UV-Vis detector. The mobile phase consisted of 20 mol L^{-1} NaH_2PO_4 aqueous solution adjusted to pH 2.5 with H_3PO_4. The column used was a C_{18} Hypersil octadecylsilane (ODS), 4.0 × 250 mm, 5 μm, with the temperature setup at 25 °C. The flow rate was 0.5 mL min^{-1} with a wavelength of 210 and 220 nm, although in both cases more sensitive signals were obtained for 210 nm. After a proper ten-fold dilution with water, injection volume was 20 μL.

The amino acids analysis, Asp, Glu, Ser, Gly, Asn, Gln, Ala, Pro, Ala-Gln and Tau, was preceded by the albumin protein removal. HSA contained in the culture medium was precipitated with ethanol as a co-solvent and then centrifuged in a microcentrifuge at 14 000 rpm for 15 min (or a relative centrifugal force (RCF) of 573 (g)). Finally, the supernatant was removed and samples were filtered through a 0.45 μm PTFE filter prior to analysis. The amino acids were chemically derivatized off column by the use of 2 μL of phenylisothiocyanate (PITC), as described by Heinrikson and Meredith;[31] the derivatisation implied incubating the samples for 30 minutes at 37 °C. In order to ensure a high recovery of amino acids and full derivatisation this step was carried out in an excess of derivatising agent. Then samples were applied to a HPLC-UV-Vis (Agilent 1100 Series) system measuring at a wavelength of 254 nm with a C_{18} Hypersil ODS column of 4.0 × 250 mm and 5 μm particle size. A gradient mode consisting of two mobile phases was used: mobile phase *A* was 50 mmol L^{-1} sodium acetate adjusted to pH 5.5 with acetic acid and *B* was acetonitrile. The column was eluted under linear gradient conditions at a constant flow rate of 1.0 mL min^{-1} as presented in Table 1. A flow of 100% acetonitrile was used during 26 minutes to wash the column and remove the unreacted PITC. After a proper dilution of 1 to 7.2, the injection volume of the derivatized sample was 5 μL.

3. Results and discussion

3.1. Assignment by ^{1}H 800 MHz NMR

Fig. 1 presents the CPMG spectra showing the metabolic profile of the IVF culture medium with several peaks derived from its complex composition. Table 2 summarises the metabolites, their chemical shifts and the assignation of the peaks for the ^{1}H NMR spectrum. Peaks for the eight amino acids: Ala, Glu + Gln, Gln,

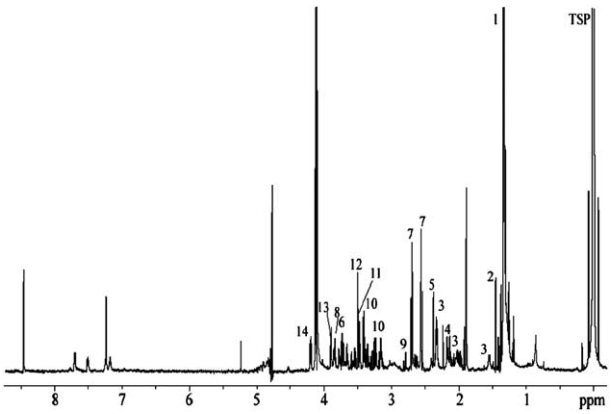

Fig. 1 Full CPMG spectra. A total of 14 metabolites can be identified: (1) lactic acid; (2) Ala; (3) Ala-Gln; (4) Glu + Gln; (5) pyruvic acid; (6) Gln; (7) citric acid; (8) Asp; (9) Asn; (10) Tau; (11) Gly; (12) glucose; (13) Ser; (14) Pro.

Table 2 Metabolites and their chemical shifts of assigned peaks for the ^{1}H NMR assignment

	Analyte	Chemical shift/ppm
	TSP	0.00
1	Lactate	4.11 (CH); 1.33 (CH_3)
2	Ala	1.45 (CH_3)
3	Ala-Gln	1.55 (CH_3); 2.00 (CH_2); 2.33 (CH_2)
4	Glu + Gln	2.15 (βCH_2)
5	Pyruvic acid	2.37 (CH_3)
6	Gln	2.40; 3.72 (αCH)
7	Citric acid	2.55 (CH_2); 2.70 (CH_2)
8	Asp	2.62 (βCH_2), 2.64 (βCH_2); 3.86 (αCH)
9	Asn	2.79 (βCH_2), 2.81 (βCH_2)
10	Tau	3.25 (CH_2); 3.41 (CH_2)
11	Gly	3.48 (αCH_2)
12	Glucose	3.46; 3.50; 3.52 (CH_2)
13	Ser	3.85; 3.91 (βCH_2)
14	Pro	4.20 (αCH)

Asp, Asn, Gly, Ser and Pro, and the dipeptide Ala-Gln and Tau were observed as well as peaks for lactic and pyruvic acids and glucose. Additionally, peaks at 2.55 and 2.70 ppm were assigned to citric acid; however no assignment for lipoic acid was possible.

NMR metabolomics is a new, fast and incredibly useful tool for obtaining the medium metabolomic fingerprint, which gives a quick idea of its profile. The NMR analyses are non-invasive as the sample is diluted with saline solution and the duration of the analysis is very short (*ca.* 9 minutes). Therefore, if a small amount of sample is acquired it can be difficult to perform enough measurements to obtain the required information without significant dilution and here, as the sample is not destroyed, it may be recovered and used in further analyses. Advantageously, in these experiments there is no need for deproteinization procedures as pulse sequences are designed for lifting the proteins. The use of an 800 MHz NMR equipment is not a common facility and it also requires high costs. Although lower field equipment (400, 500 and 600 MHz) can be used, plenty of disadvantages appear as acquisition times grow, higher sample volumes are needed and resolution power decreases.

3.2. Determination of glucose by HPLC-MS

We first consider the determination of glucose in IVF culture media. Simultaneous detection of glucose together with the organic acids (lactic and pyruvic) determination was not viable as glucose concentrations were below the detection limit; glucose adsorption wavelength is below 210 nm and additionally has a very small extinction coefficient. However, HPLC coupled to a refractory index detector (HPLC-RID) is an established technique for the determination of sugars in different samples.[32,33] However, our preliminary determination of glucose using HPLC-RID presented one main drawback related to interferences of HA, lactic and pyruvic acids present in the culture medium with retention times close to that for glucose. Consequently, our glucose analyses turned out to be relatively higher than previously reported.[2] Therefore, mass spectrometry was used to determine glucose concentrations as a precise and selective technique for metabolomics studies.

Fig. 2 depicts the mass spectrum for the culture medium analysed. A positive ionization polarity was chosen and three adducts were detected, $+H^+$ ($m/z = 181$), $+Na^+$ ($m/z = 203$), and $+K^+$ ($m/z = 219$) but overall the most abundant target mass and therefore the selected one was that corresponding to the 203-mass respective to the [Glucose + Na]$^+$ adduct. The glucose retention time for these conditions was 11.4 minutes and a linear calibration curve in ultra pure H_2O over a range of 0.028–0.389 mmol L^{-1} was obtained with a slope of 168.067 $mmol^{-1}$ L ($R^2 = 0.9996$). The final concentration of glucose of this IVF medium was calculated to be 0.461 ± 0.004 mmol L^{-1} (in all cases using Student's-t for 95% probability and a sample of $n = 8$) from the calibration plots and achieved a detection limit of 13 μM and a limit of quantification of 44 μM.

Attempts on the direct infusion to the mass spectrometer of the IVF medium were contemplated as a feasible analytical approach in order to decrease the analysis time. Although we diluted the sample with water, the high osmolality (between 270 and 290 mOsm in physiological media) provoked capillary blocking. Hence, the use of a carbohydrate HPLC column benefited the separation of salts and complex analyte mixtures and we could target glucose with no need for deproteinization. Once glucose

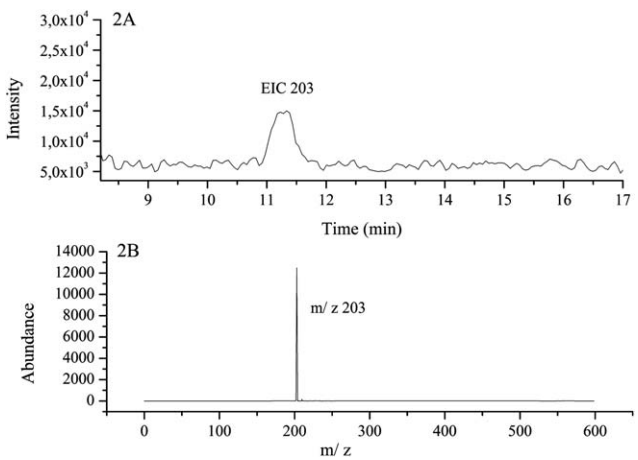

Fig. 2 Zoom of the chromatogram (A) and mass spectrum (B) for glucose determination in the IVF culture media.

was separated, this was identified and quantified with the mass spectrometer following either scan mode or secondary ion mass spectrometry (SIM) mode. Within a scan range between 35 and 500 *m*/*z* no additional carbohydrates were found. Furthermore, SIM mode was used successfully for the quantification of the sodium–glucose most abundant adduct with a higher sensitivity.

Glucose determination can be also followed by enzymatic assay using ultramicrofluorescence where glucose is first converted *via* enzymatic catalysis to glucose-6-phosphate in the presence of adenosine-5′-triphosphate (ATP).[13] Further, the latter compound is converted enzymatically to 6-phosphogluconolactone with the additional production of the nicotinamide adenine dinucleotide phosphate (NADPH). Although this fluorimetric method requires a small portion of the sample, 1–2 μL, the protocol involves generally extensive analyses time. Different strategies for the determination of glucose in cell culture or biological fluids are now emerging. Electrochemical devices and microdevices focused on the monitoring of different analytes involved in cell growth and this development has been commercialised. For example, Pereira and collaborators measured the real-time monitoring of glucose in cell culture by using microfluidic electrochemical biochips.[34] Nevertheless, the rapid determination of glucose with an electrochemical commercial device commonly used by diabetic people was unfeasible and the authors speculate that the surface was quickly blocked.

3.3. Determination of lactic acid and pyruvic acid by HPLC-UV-Vis

We now turn towards the analysis of lactic and pyruvic acids in the IVF culture medium utilising a HPLC-UV-Vis detector. Fig. 3 depicts a typical chromatogram arising from the IVF culture medium at a dilution of 1 to 10 in phosphate buffer solution pH 7.0 for an injection volume of 20 μL. As depicted in Fig. 3, peak A corresponds to pyruvic acid and peak B to lactic acid, based on comparison with analytical standards. Calibration curves for pyruvic acid were obtained in an aqueous solution consisting of 20 mM phosphate buffer pH 2.5 and measured at 210 nm wavelength which exhibits a retention time of 5.69 minutes. A linear response to increasing concentrations of pyruvic acid over a range of 0.005–0.1 mmol L^{-1} was obtained with a slope of 1276.2 $mmol^{-1}$ L ($R^2 = 0.9998$). Lactic acid was found to exhibit a retention time of 7.28 minutes giving a linear response over the concentration range studied, 0.1–2 mmol L^{-1}, with a slope of 77 $mmol^{-1}$ L ($R^2 = 0.9999$). The chromatographic characterisation for pyruvic and lactic acids determination was: the resolution factor (R_s) was 6.564, the capacity factor (k') of pyruvic acid was 0.317 and for lactic acid 0.671, and the selectivity factor (α) for the two peaks was found to be 2.116. The analytical utility of the HPLC for the quantification of these two organic acids has been proven and at concentrations of 0.395 ± 0.008 mmol L^{-1} (*Student's-t* 95%, $n = 8$) of pyruvic acid and 19.9 ± 0. 3 mmol L^{-1} (*Student's-t* 95%, $n = 8$) of lactic acid has been calculated for the culture medium from the calibration plots, showing detection limits / quantification limits of 28 / 92 μM and 1.2 / 3.9 mM, respectively.

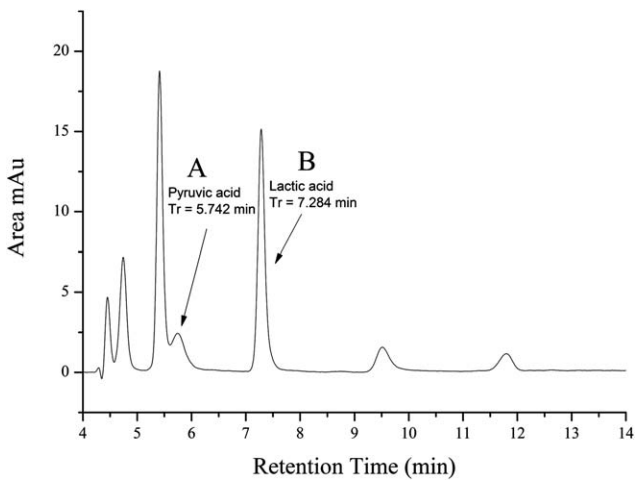

Fig. 3 Typical chromatogram of the IVF culture medium clearly demonstrating the eluted peaks of pyruvic acid ($t_r = 5.742$ min) and lactic acids ($t_r = 7.284$ min).

Despite HSA being present at a high concentration in the culture medium *ca.* 5 mg mL^{-1} deproteinisation was not required for the inter-day determination of both organic acids. Furthermore and as previously mentioned in the Experimental section, the only sample preparation which needed deproteinisation with ethanol was the determination of the amino acids. The effect of pH in the range between 2.0 and 3.0 was evaluated to observe the potential effect on the resolution of the pyruvic acid peak but a pH of 2.5 was found to be the optimum pH. Rapid determination of lactic and pyruvic acids was achieved in 8 minutes without further purification or elimination of macromolecules such as HA and HSA.

Pyruvate has been commonly investigated *via* fluorescence methods, electrochemical detectors coupled with HPLC, spectroscopic methods or enzymatic reactions. Wulkan and coworkers used HPLC coupled to a fluorescence detector to analyse pyruvate in a complex matrix of blood.[35] Recently, Wolff[36] and collaborators compared HPLC with two detection methods: fluorescence *versus* enzymatic assay to measure pyruvate in blood in clinical practices, taking into account that the fluorescence method required a derivatisation step. Alternatively, the enzymatic assay is based on the reduction of pyruvate to lactate by lactate dehydrogenase at pH 7.5 with the nicotinamide adenine dinucleotide (NADH) in excess. The concomitant oxidation of NADH is then measured by fluorimetric detection[13] or spectrophotometrically at 340 nm. The latter procedure presents several drawbacks since the recovery can be insufficient and therefore is considered as estimation for pyruvate in blood.[37]

HPLC-RID has also been postulated as an analytical option for lactate determination in a wide variety of media such as blood plasma, serum, and wine.[38] Likewise, electrochemical and fluorimetric detectors are also commonly used for the determination of lactate of different media natures.[39,40] On one hand, electrochemical detectors require working at isocratic flow as well as relative conductive mobile phases, presenting a main drawback and passivating carbonaceous electrodes.[41] On the other hand, the use of microfluorimetric detectors requires derivatisation of most samples.[42]

Here, the rapid determination of lactic and pyruvic acids concentration is crucial as this allows us to determine the lactate to pyruvate ratio in this culture medium, which is biologically significant as this reflects the cellular NADH to NAD^+ ratio.

This factor named the *relative redox potential* can be used as an indicator of the redox status and the kinetics of the embryo development.[43]

3.4. Determination of amino acids, taurine and alanine-glutamine by HPLC-UV-Vis

Fig. 4 depicts the response for eight amino acids identified in the IVF culture media, Asp, Glu, Ser, Gly, Asn, Gln, Ala, and Pro, the dipeptide Ala-Gln and Tau, when analysed by HPLC-UV-Vis at 254 nm. Table 3 presents the correlation of the peak number (N), the analyte assignment, and the retention time (t_r) as well as the calibration curve slopes (a), the y-intercept value (b) and its regression coefficients (R^2) over a studied range of 0.028–0.280 mmol L^{-1} for the cited amino acids.

Accordingly, the concentration of each analyte in the IVF culture medium with this method was calculated from the calibration plots to be Asp 0.670 ± 0.002 mmol L^{-1}, Glu 0.540 ± 0.001 mmol L^{-1}, Ser 0.565 ± 0.003 mmol L^{-1}, Gly 0.2616 ± 0.0004 mmol L^{-1}, Asn 0.3879 ± 0.0005 mmol L^{-1}, Gln 0.1070 ± 0.009 mmol L^{-1}, Tau 0.364 ± 0.001 mmol L^{-1}, Ala 0.880 ± 0.003 mmol L^{-1}, Pro 0.2734 ± 0.0006 mmol L^{-1} and Ala-Gln 0.570 ± 0.001 mmol L^{-1} (in all cases using *Student's-t* for 95% probability and a sample of $n = 7$). The detection / quantification limits achieved with this separation totally assure the suitability of this technique for the analysis of this complex medium, as referred before, and following the same order of appearance as they were: 8 / 27 μM for Asp, 4 / 14 μM for Glu, 9 / 30 μM for Ser, 1 / 4 μM for Gly, 2 / 5 μM for Asn, 30 / 102 μM for Gln, 3 / 12 μM for Tau, 10 / 32 μM for Ala, 2 / 7 μM for Pro and finally, 4 / 12 μM for Ala-Gln.

As mentioned in the Experimental section, the deproteinization step was essential in the amino acids determination, so it was carried out using ethanol as co-solvent. It is well known that PITC can react with *N*-terminus of proteins as well as amino groups of lateral chains in amidic amino acids.[31] There are two types of amino acids, essential and non-essential, and depending on the embryo developmental stage they could be taken in by cells, where they undergo further degradation, *i.e.* catabolic processes, or used as a nitrogen source. The uptake and depletion of amino acids are related to the growth and development of human embryos.[14]

3.5. Analysis of dataset

In this step now we turn to summarise the results obtained from the four determinations in order to get insight into the nature of the culture medium, the time of analysis and the feasibility of the measurements. Fig. 5 shows the global scheme for a complex medium analysis. This figure emphasizes on the analytical platform developed, where generally in IVF the sample volumes are between 20 and 30 μL after human embryo retrieval. The first step tackles the qualitative and fingerprint analysis of the sample, which does not require any chemical modification. Thereafter, the whole sample could be redirected to the chromatographic measurement for the quantitative determination of analytes, in

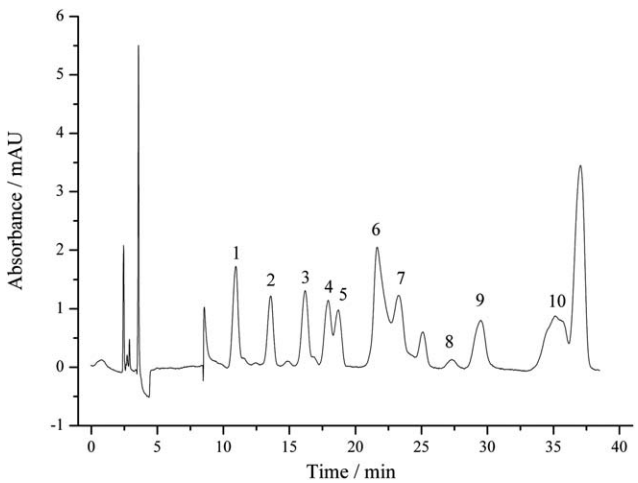

Fig. 4 Zoom of the chromatogram of the IVF culture medium showing the eluted peaks of the amino acids: (1) Asp; (2) Glu; (3) Ser; (4) Gly; (5) Asn; (6) Gln; (7) Tau; (8) Ala; (9) Pro; and (10) Ala-Gln.

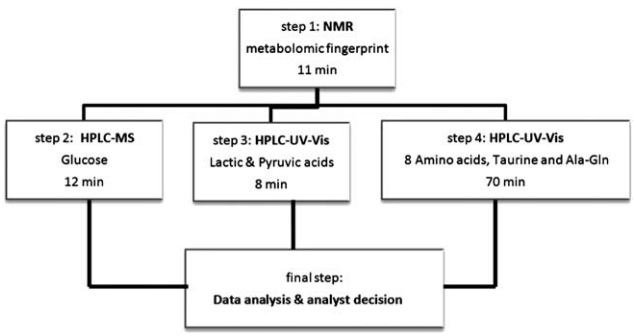

Fig. 5 Scheme of the steps required for a completion of the combinatorial analytical platform.

Table 3 Amino acids analysis parameters

N	Analyte	t_r/min	a/mmol^{-1} L	b	R^2	k'	α
1	Asp	10.95	939.219	12.649	0.9901	3.380	1.311
2	Glu	13.58	994.09	19.655	0.9803	4.432	1.238
3	Ser	16.22	1075.040	16.610	0.9852	5.488	1.127
4	Gly	17.96	3437.387	52.219	0.9849	6.184	1.050
5	Asn	18.74	1371.424	20.155	0.989	6.496	1.155
6	Gln	21.26	1156.226	17.352	0.9877	7.504	1.109
7	Tau	23.30	2597.375	26.857	0.9914	8.320	1.192
8	Ala	27.29	694.865	12.743	0.9821	9.916	1.090
9	Pro	29.52	4980.587	37.885	0.9912	10.808	1.209
10	Ala-Gln	35.18	3783.921	36.560	0.9902	13.072	−0.076

this case organic acids and glucose. The amino acids determination step is placed at the end of this platform since it involves the chemical modification of the sample. In conclusion, the analytical platform manages the identification and quantification of the sample and provides the analyst with the metabolomic profile of the micro IVF fluid after retrieval of embryos. Furthermore, this analytical platform could be applied to many kinds of culture media commonly used. The analytical platform requires minimal chemical modification and furthermore it involves easy sample manipulation. Overall this has important consequences for the data discussion and the final decision of the embryologist.

4. Conclusions

The objective of this study was to determine the composition of the commercial embryo culture medium. We have shown the versatility of HPLC-UV-Vis, HPLC-MS and ^{1}H NMR spectroscopy as well as the appropriate procedure optimisation in order to analyse as many analytes as possible, present in 20–30 μL of real sample after embryo development. Although the presence of interferences such as proteins and glycosaminoglycans and the high level of osmolality of the IVF media, just a conditioning procedure based on a derivatisation step, was required for the amino acids analysis. Additionally, we exploit the LC as a routine, easy and accessible technique for non-expert technicians at chemical and biological laboratories, and the NMR as a powerful technique which contributes simultaneously to both determine new molecules and provide the whole metabolomic profile of the culture medium.

A rapid composition determination could be achieved relatively fast as the analysis takes no longer than 12 minutes for glucose mass determination, 8 minutes for pyruvic and lactic acids quantification and a complete separation of ten amino acids in 70 minutes. Furthermore, we show here the possibility of obtaining an NMR fingerprint by screening the global sample composition in order to get a glance at it in just 11 minutes which is not possible with any chromatography. This combinatory approach provides a versatile methodology which would help the embryologist in the evaluation or assessment process of the embryo viability.

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