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Mass spectrometry fingerprinting of media used for *in vitro* production of bovine embryos

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Using the bovine species as a biological model, direct infusion chip-based nano-electrospray ionization mass spectrometry (nano-ESI-MS) fingerprinting in the positive ion mode is used to obtain fast chemical profiles of media used for *in vitro* production of bovine embryos. Nano-ESI-MS fingerprinting is useful for characterization and routine quality control requiring no sample pre-separation, being able to differentiate four different media (IVM, IVF, SOF and HSOF) via principal component analysis (PCA). For media stored at +4°C for up to 45 days, no significant ($p > 0.05$) variation was observed in cleavage and blastocyst rate development, as well as in the nano-ESI-MS chemical profiles. For media exposed to a heat shock (60°C for 3 h), no significant decrease ($p > 0.05$) in embryo development rates was observed, but nano-ESI-MS profiles were quite distant from fresh control media in the PCA. For frozen media (−70°C for 2 months), again no significant variation ($p > 0.05$) in embryo development was noticed, but nano-ESI-MS profiles from all media were significantly affected. These results indicate that nano-ESI(+)-MS fingerprinting was able to characterize different media based on their specific chemical profile. The technique seems therefore applicable as a routine quality control assay, detecting, for example, compositional changes after temperature variations that may affect post-transfer embryo viability. Copyright © 2009 John Wiley & Sons, Ltd.

Bovine embryo *in vitro* production (IVP) allows a significant increase in the number of offspring per animal. This biotechnology is widespread, especially in Brazil, mainly due to the reproductive physiology of the Nellore cattle breed, owing to its commercial and genetic value.[1] Embryo IVP is also the basis for other biotechnologies such as animal cloning and trangenesis.[2]

For bovine embryo IVP, media quality is fundamental for satisfactory results. Quality variation due to degradation during transportation and storage or technical failures during preparation may cause extensive losses. Quality evaluation of media used for commercial IVP of bovine embryos is usually performed by measuring the pH, the osmolarity and evaluating the rates of cleavage and blastocyst *in vitro* development. These parameters may fail to reveal suboptimal media conditions, which could have a low impact on embryo development rates, but could affect embryo post-transfer survival. For human-assisted reproduction, the efficiency of assay protocols for *in vitro* fertilization (IVF), embryo handling and *in vitro* culture media quality evaluation is under discussion and there is a strong recommendation that results from the mouse embryo assay (MEA) be associated to other assays using cells or spermatozoa due to inherent variation found in biological assays.[3–5]

Mass spectrometry (MS) is a powerful analytical technique able to characterize and quantify a wide range of elements, molecules and biomolecules present in pure forms or in complex mixtures. Initially, MS was restricted to the analysis of gaseous, volatile and thermally stable compounds because of the limitations of electron and chemical ionization. Applications of MS have been greatly extended, however, after the development of 'soft' ionization techniques such as matrix-assisted laser desorption/ionization (MALDI)[6] and electrospray ionization (ESI),[7] which now allow handling of polar, large and even loosely bonded molecules. In the biotechnology field, MALDI-MS has been applied in

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proteomics studies and for biomarker characterization.[8–10] In embryology, MS has been already employed, for instance, to expand knowledge of mammalian embryo metabolism.[11]

Via ESI, molecules of a large range of polarity and mass can be transferred directly to the gas phase in intact forms, such as the protonated or cationized molecules. One interesting application of ESI-MS is to obtain characteristic chemical profiles (fingerprints) directly from complex mixtures with no pre-separation or sample preparation steps. ESI-MS fingerprinting has been used therefore to characterize, classify, control product quality, probe authenticity, monitor adulteration and evaluate the stability of various products. We have, for instance, applied ESI-MS fingerprinting for perfumes,[12] beverages,[13–17] vegetable oils,[18] biodiesel,[19] and snake venoms.[20]

The aim of this work was therefore to test the ability of ESI-MS to provide reliable fingerprinting characterization of media for embryo production used in *in vitro* production (IVP) of bovine embryos as a proof-of-principle case. Direct infusion nano-ESI-MS was used, and the fingerprinting data was tested for characterization, evaluation of storage conditions and detection of chemical changes after significant temperature variations.

EXPERIMENTAL

Chemical reagents and samples

For the nano-ESI-MS experiments, all reagents used were of analytical grade. For embryo production experiments, unless mentioned otherwise, chemicals and growth media were purchased from Sigma-Aldrich (St. Louis, MO, USA). For all experiments, incubator conditions for oocytes and embryos were maintained at 38.5°C with 5% CO_2 in air for *in vitro* maturation (IVM) and *in vitro* fertilization (IVF); and with atmosphere conditions of 5% CO_2, 5% O_2 and 90% N_2 for *in vitro* culture (IVC), with maximum humidity.

General experimental procedures

Four media employed for IVP of bovine embryos were used in the experiments: (i) *in vitro* maturation medium (IVM-medium); (ii) *in vitro* fertilization medium (IVF-medium), (iii) synthetic oviduct fluid medium (SOF-medium), and (iv) HEPES-buffered synthetic oviduct fluid medium (HSOF-medium). Media batches were used to produce embryos and to acquire nano-ESI-MS fingerprints. Experimental design aimed to characterize the chemical profile of (i) media during a period of 6 weeks under storage at +4°C, (ii) media after exposure to a heat shock (+60°C for 3 h), and (iii) media after 2 months of storage at −70°C. All embryo production experiments were performed with at least three replicates.

For embryo IVP, oocytes were obtained *post mortem* by follicular aspiration of ovaries from cows slaughtered at a local abattoir. The ovaries were transported in 0.9% saline solution (w/v) at 25–30°C to the laboratory. Follicles with diameters between 3 and 8 mm were aspirated using an 18-gauge needle attached to a 20-mL syringe. *Cumulus* oocyte complexes (COCs) with at least three layers of *cumulus* cells and homogeneous cytoplasm were washed in HEPES-buffered tissue culture medium 199 (TCM-199; GIBCO BRL®, Grand Island, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), as well as with 0.20 mmol L^{-1} sodium pyruvate and 83.4 μg/mL amicacin. Groups of up to 20 COCs were placed in 100 μL of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 0.20 mmol L^{-1} sodium pyruvate, 83.4 μg/mL amicacin, 1 ng/mL FSH (Pluset, Laboratórios Calier do Brasil Ltda, Brazil), 50 μg/mL LH (Vetecor, Laboratórios Calier do Brasil Ltda, Brazil), and 1 μg/mL estradiol under mineral oil during 24 h for IVM. Oocytes were *in vitro* fertilized in TALP-IVF (Tyrod's albumin lactate and pyruvate – *in vitro* fertilization) medium supplemented with 0.6% (w/v) bovine serum albumin (BSA), 30 μg/mL heparin, 18 μmol L^{-1} penicillamine, 10 μmol L^{-1} hypotaurine and 1.8 μmol L^{-1} epinephrine. Motile thawed spermatozoa from one bull were centrifuged (500 *g* for 5 min) twice in 2 mL of TALP medium supplemented with 0.2 mmol L^{-1} sodium pyruvate, 83.4 μg/mL amicacin and buffered with 10 mmol L^{-1} HEPES. The final sperm concentration was 2.5×10^6 live spermatozoa/mL. Sperm from the same bull was used for all experiments. Oocytes and sperm were incubated for 22–24 h. After incubation, presumptive zygotes were stripped from *cumulus* cells and spermatozoa by gentle pipetting and were cultured in SOF-medium[21] supplemented with 0.6% (w/v) BSA and 2.5% (v/v) FCS. Embryo cleavage was evaluated 48 h post-insemination, and embryo development at day 7 of IVC. After evaluation, embryos were loaded into 0.25-mL straws with HSOF-medium for 3 h at 36°C to simulate transport for embryo transfer. After this period, embryos were placed back in the same culture dish and evaluated for blastocyst hatching after 24 h.

Blastocyst rate is given as the number of total oocytes submitted to IVF/number of embryos at day 7 of IVC. Blastocyst hatching rate is given as the number of hatched blastocysts at day 8/number of embryos at day 7 of IVC.

For direct infusion automated chip-based nano-ESI-MS[22] in the positive ion mode, media samples were diluted 1:100 with ultrapure water and loaded into 96-well plates (total volume of 100 μL in each well). A quadruple time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) equipped with a Triversa NanoMate 100 (Advion BioSciences, Ithaca, NY, USA) was used. General conditions were: gas pressure, 0.3 psi; capillary voltage, 1.55 kV; and cone voltage, 49 V. Mass spectra were accumulated over 60 s, centered and aligned using the MassLynx 4.0 software (Waters, Manchester, UK).

Data handling

Cleavage, embryo development and embryo hatching percentage values were submitted to analysis of variance (ANOVA) using SAS System software v.9.1 (SAS Institute Inc., Cary, NC, USA). Mean values were compared by Tukey's test, with significance level of 5%.

From the nano-ESI-MS data, a matrix built with relative ion intensities for ions ranging from *m/z* 50 to 1000 was used for principal component analysis (PCA) using Pychem 3.0.2v.[23] To compare *m/z* profiles from various spectra at the same time, heat map graphics[24] were adapted from the 'Heat Map Viewer' tool – Gene Pattern.[25]

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Table 1. Results of bovine embryo *in vitro* production using fresh media (day 0), and the same batch stored for 30 days (day 30) and 45 days (day 45)

Group	No. ocytes	% Cleaved (SD)	% Blast* (SD)	% Hatched blast** (SD)
Day 0	928	71.5[a] (5.2)	34.9[a] (4.7)	53.8[a] (8.0)
Day 30	357	76.5[a] (5.2)	39.5[a] (14.2)	56.3[a] (6.6)
Day 45	338	78.0[a] (3.1)	40.8[a] (5.63)	63.6[a] (18.1)

[a] Same letter indicates statistical similarity ($p > 0.05$).
* Blastocysts (Day 7 of *in vitro* culture).
** Hatched blastocysts (Day 8 of *in vitro* culture).

RESULTS

Four media used to produce bovine embryos (IVM, IVF, SOF and HSOF) prepared on the same day were considered to be of the same batch. Polypropylene tubes were used to store media. These tubes were maintained at +4°C, pH 7.2–7.4, with an atmosphere of 10% CO_2 and 90% N_2 inside the tubes. Bovine embryos were produced using different media batches starting at the day of media preparation (day 0), after 30 days of storage (day 30) or after 45 days of storage (day 45), in four replicates. Table 1 summarizes the results of embryo development when day 0, day 30 and day 45 media batches are compared. No negative effects on embryo cleavage, blastocyst and hatching percentages were noted among media batches from day 0, day 30 and day 45 ($p > 0.05$).

Because experimental day-related variation due to animal breed, nutrition and age may occur when oocytes obtained from ovaries of slaughtered cows are used, we also compared embryo development between day 30 and the day 0 media batches, as well as between day 45 and day 0 media batches on the same experimental day.

When day 30 and day 0 batches used on the same experimental day (three replicates) were compared, no differences were observed ($p > 0.05$) in cleavage (79.7 and 76.5% for control and day 30 media, respectively) and in blastocyst percentages (40.4 and 39.5% for control and day 30 media, respectively). Nonetheless, blastocyst hatching percentage in day 30 media was lower (84.7 and 56.3% for control and day 30 media, respectively; $p < 0.05$).

When day 45 and day 0 batches used on the same experimental day (three replicates) were compared, day 45 media presented similar percentages of cleavage (80.8 and 78.5% for control and day 45 media, respectively), blastocyst (36.1 and 40.8% for control and day 45 media, respectively) and blastocyst hatching (73.4 and 66.3% for control and day 45 media, respectively; $p > 0.05$).

Nano-ESI-MS fingerprints (Fig. 1(a)) from the four different media used for bovine embryo production were subjected to PCA (Figs. 1(b) and 1(c)). Comparing, at the same time, six different batches of each medium stored for 1 to 6 weeks (Fig. 1(b)), only HSOF-medium batches were grouped and separated from the three others (86.1% of the variance explained). When the IVM-, IVF- and SOF-medium samples were compared in a second PCA (Fig. 1(c)), three isolated groups were observed, with 83.6% of the variance explained. The detection or absence of ions and their abundance differences among spectra (Fig. 1(a)) can be visualized also by using the heat map approach (Fig. 1(d)). The HSOF-medium displays a unique spectrum, in which the ion of m/z 261 is the most intense. This ion likely corresponds to the monoprotonated sodium HEPES.

Some ions, which are putatively assigned below, indicate that individual media components may be monitored via the ESI-MS fingerprints: m/z 129 [sodium pyruvate + H_2O + H]$^+$, m/z 135 [lactic acid + Na]$^+$, m/z 165 [L-glutamine + H_2O + H]$^+$, m/z 175 [L-arginine + H]$^+$, m/z 203 [myo-inositol + Na]$^+$, m/z 219 [myo-inositol + K]$^+$, and m/z 394 [phenol red + K]$^+$.

Since media stored at +4°C showed no significant variation either biologically or in its ESI-MS fingerprints, we exposed samples to a temperature of +60°C for 3 h to verify if a heat-shock treatment would affect embryo development and the nano-ESI-MS profile. For embryo development rates, compared to control (total of 295 oocytes), heat-shock media (total of 268 oocytes) presented similar cleavage (77.4% and 65.4% for control and heat-shock media, respectively; $p > 0.05$), blastocyst development (19.5 and 16.9% for control and heat-shock media, respectively) and blastocyst hatching percentages (75.4 and 84.6% for control and heat-shock media, respectively).

PCA of the nano-ESI-MS fingerprints from the media submitted to heat shock compared to the media stored at 4°C for up to 6 weeks (Figs. 2(a)–2(c)) placed the heat-shock treatment sample distant from the other non-treated samples (with 93.04, 85.62 and 83.54% of data variance explained, respectively). Some ions displayed significant abundance variations (e.g. m/z 204 and 302), some new ions appeared (e.g. m/z 78, 180, 222 and 246), and some ions disappeared (e.g. m/z 161, 258, 265, 394 and 395; Fig. 2(d)).

Besides storage at +4°C, storage at −70°C is an interesting process to evaluate via ESI-MS fingerprinting since freezing could allow extended periods of media storage. For the biological assay, four batches of fresh media were used for embryo IVP. The same batches were then thawed after 2 months and used again for embryo IVP (total of 854 oocytes for fresh and 380 oocytes for frozen media). Cleavage (71.6 and 76.9% for fresh and frozen media, respectively) and blastocyst percentages (34.9 and 36.2% for fresh and frozen media, respectively) were similar ($p > 0.05$) for the fresh media and for the media stored at −70°C. Blastocyst hatching percentage (53.8 and 79.4% for fresh and frozen media, respectively), however, was superior ($p < 0.05$) for frozen media. The PCA of nano-ESI-MS data from the four media evaluated showed that the ion profiles of frozen samples were significantly different from control samples (Figs. 3(a)–3(d)). Analysis of the heat maps (Fig. 3(e)) indicates ions (m/z values) that appeared, disappeared, and varied significantly in abundance (Table 2).

DISCUSSION

In this study, as a proof-of-principle example, ion profiles of direct infusion nano-ESI-MS fingerprints of media used for the *in vitro* production (IVP) of bovine embryos were obtained and compared with embryo biological assay

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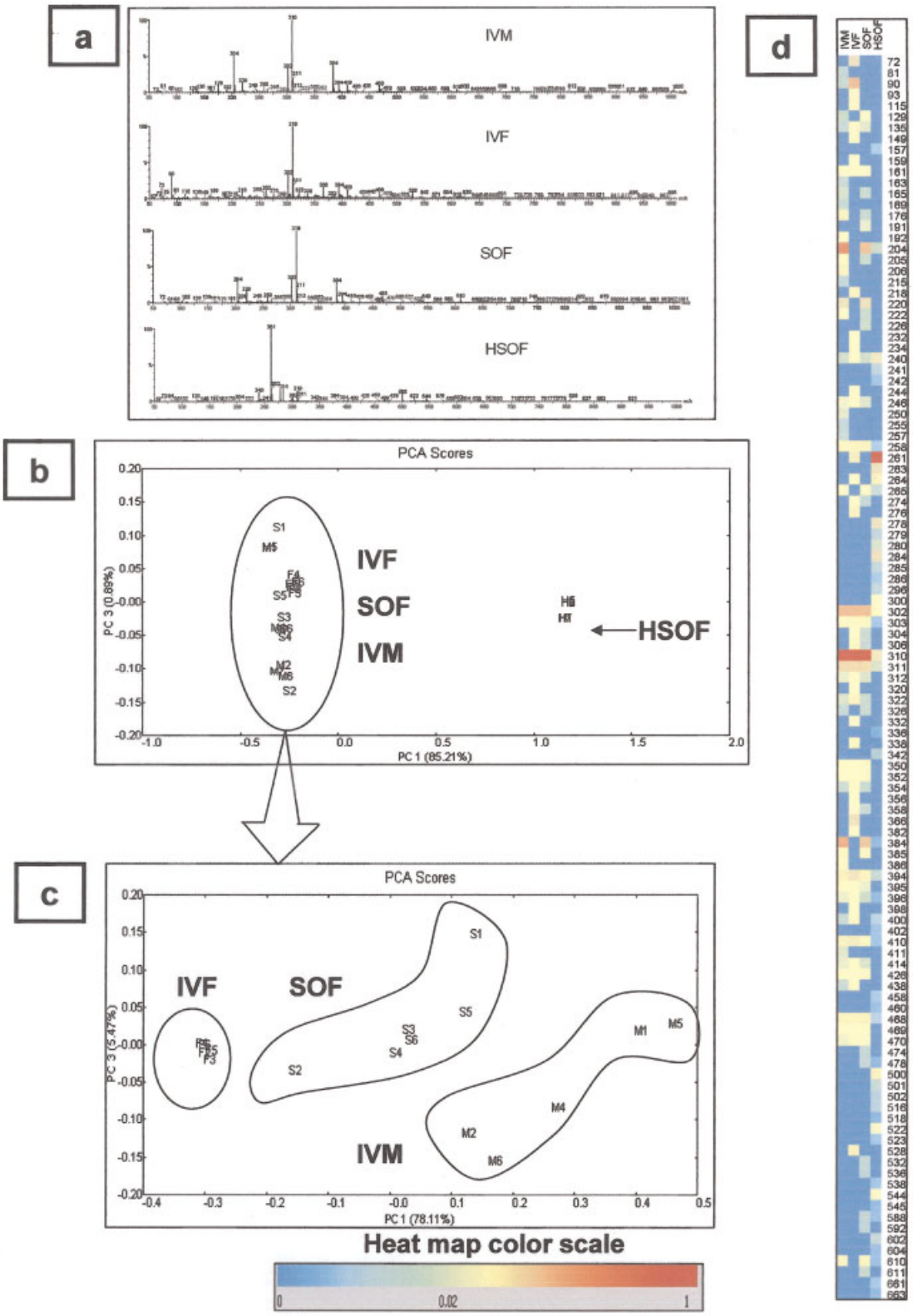

Figure 1. (a) Nano-ESI-MS fingerprints of the four different fresh media used in this study: *in vitro* maturation (IVM), *in vitro* fertilization (IVF), synthetic oviduct fluid (SOF) and HEPES-buffered synthetic oviduct fluid (HSOF). (b) The first principal component analysis (PCA) of the nano-ESI-MS data for samples of each medium ('M' stands for IVM, 'F' stands for IVF, 'S' stands for SOF, and 'H' stands for HSOF medium) stored at +4°C for 1 to 6 weeks was able to separate the HSOF medium. (c) A second PCA including just IVM, IVF and SOF samples was able to group samples from the same medium. (d) The heat map with *m/z* values obtained from the four fresh media is another way of comparing spectra shown in (a). This figure is available in color online at www.interscience.wiley.com/journal/rcm.

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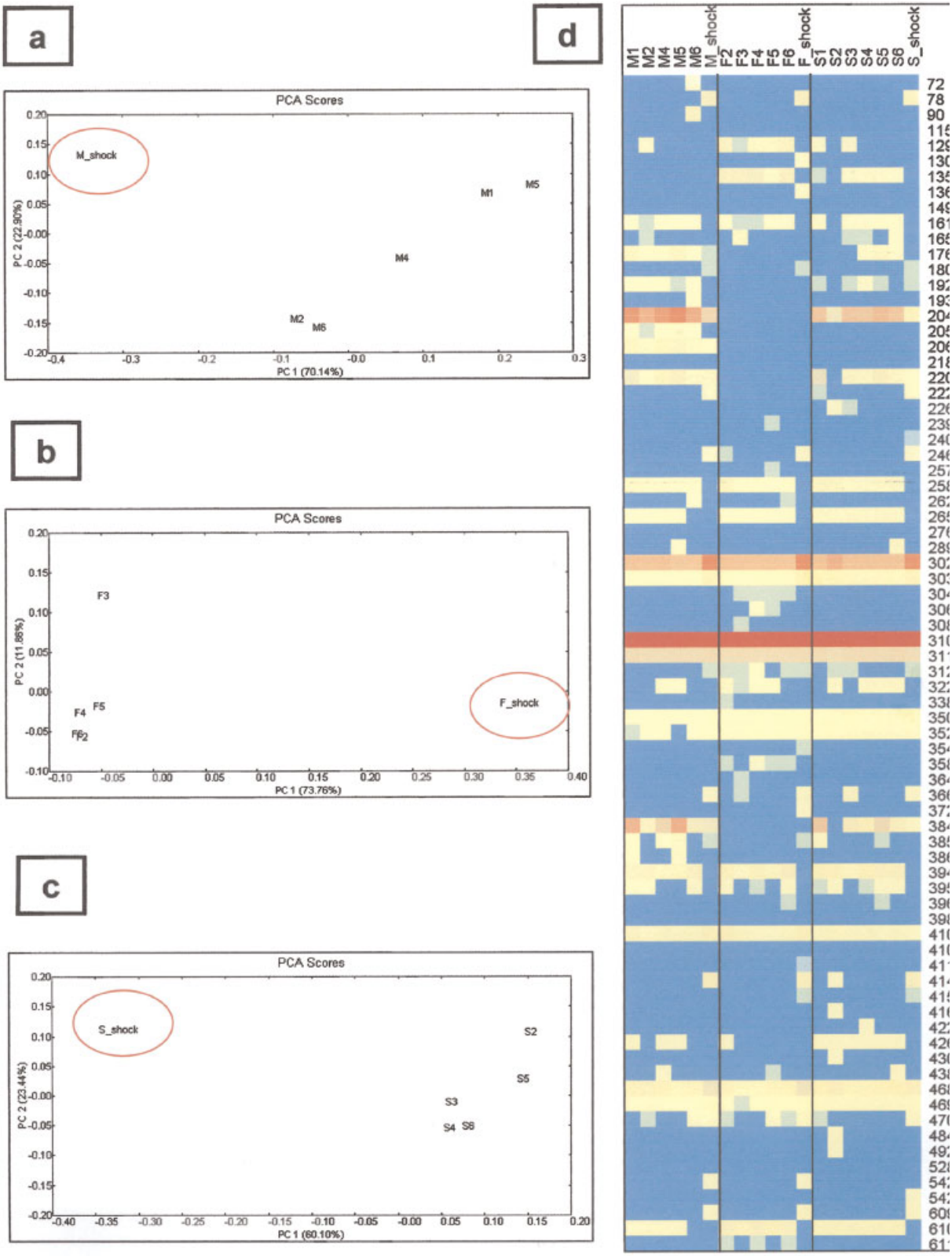

Figure 2. Comparison of media samples stored at +4°C, with one batch exposed to heat shock, using PCA and heat map. (a) PCA of IVM-medium samples stored at +4°C (identified as M1, M2, M4, M5 and M6) and the batch submitted to heat shock (identified as M_shock). (b) PCA of IVF-medium samples stored at +4°C (identified as F2, F3, F4, F5 and F6) and the batch submitted to heat shock (identified as F_shock). (c) PCA of SOF-medium samples stored at +4°C (identified as S2, S3, S4, S5 and S6) and the batch submitted to heat shock (identified as S_shock). (d) Heat map with *m/z* values of all samples, where is possible to notice ion intensity and compositional changes among spectra of the three different media. This figure is available in color online at www.interscience.wiley.com/journal/rcm.

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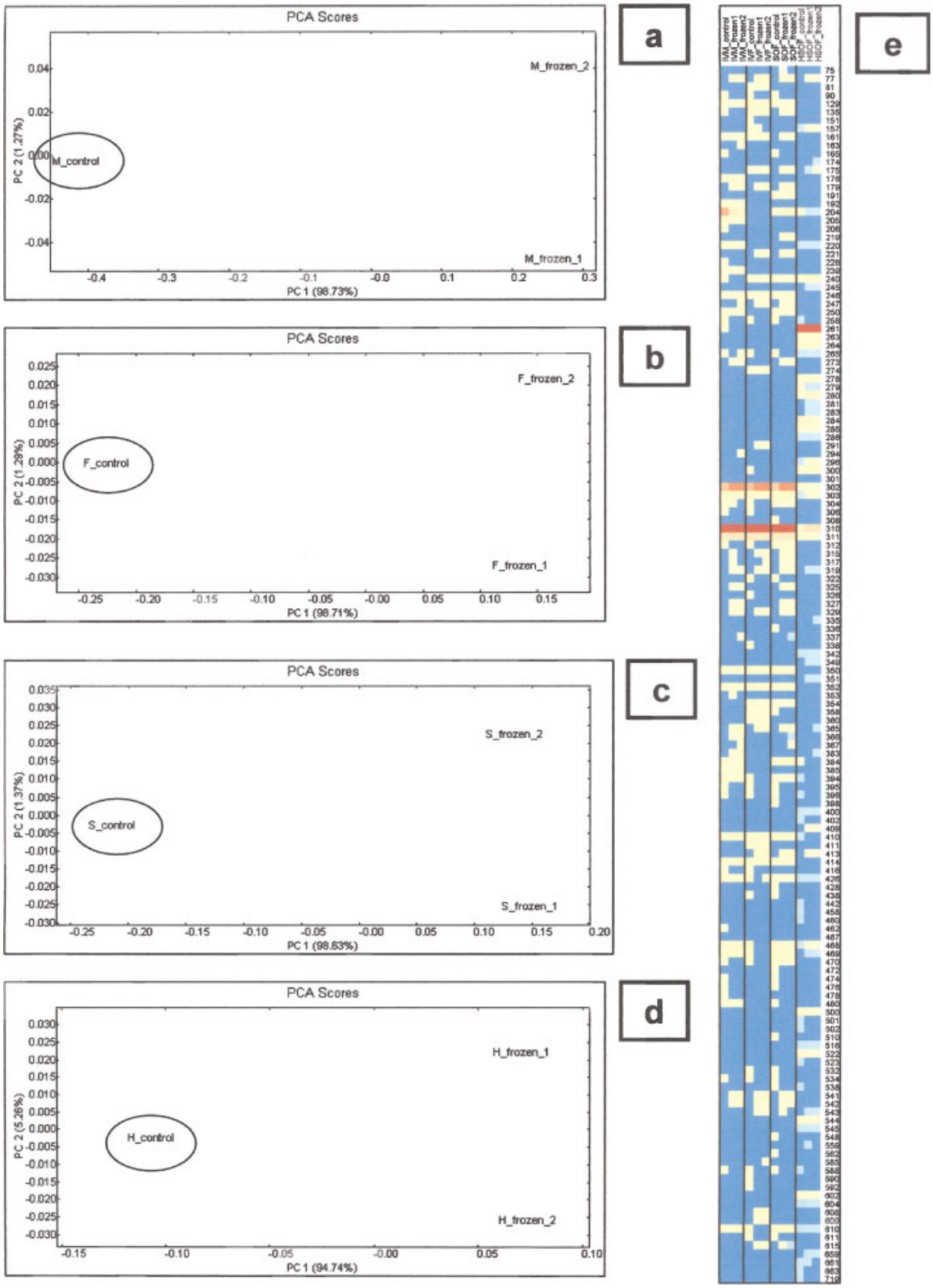

Figure 3. PCA of fresh media and two batches of frozen media stored at −70°C and during 2 months for the four media used in the experiments. (a) PCA of one sample of fresh IVM-medium (identified as 'M-control') and two batches of frozen media (identified as 'M_frozen_1' and 'M_frozen_2'). b) PCA of one sample of fresh IVF-medium (identified as 'F-control') and two batches of frozen media (identified as 'F_frozen_1' and 'F_frozen_2'). (c) PCA of one sample of fresh SOF-medium (identified as 'M-control') and two batches of frozen media (identified as 'S_frozen_1' and 'S_frozen_2'). (d) PCA of one sample of fresh HSOF-medium (identified as 'H-control') and two batches of frozen media (identified as 'H_frozen_1' and 'H_frozen_2'). (e) The heat map with *m/z* values from all samples, where it is possible to compare ion intensities and compositional differences between fresh and frozen samples. This figure is available in color online at www.interscience.wiley.com/journal/rcm.

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Table 2. Ions (*m/z*) in the ESI-MS fingerprints that appeared, disappeared, showed significant abundance variation or remained practically unaffected for frozen media

	IVM	IVF	SOF	HSOF
Appeared	77, 179, 273, 319, 325, 327, 329, 365, 366	77, 179, 221, 247, 291, 315, 319, 329, 365, 413, 541, 608	77, 135, 161, 175, 179, 219, 221, 273, 315, 317, 325, 365, 413, 542	77, 245, 281, 283, 296, 409, 413
Disappeared	90, 135, 165, 240, 258, 262, 395, 396	90, 151, 258, 265, 300, 304, 306, 322, 326, 338, 394, 416, 467, 532, 588	90, 165, 258, 265, 308, 322, 336, 358, 396, 428, 438, 472, 480, 510, 534, 562, 588	279
Abundance variation	204, 302	129, 135, 302	302, 394	157, 204, 280, 302, 310, 468
No variation	192, 205, 220, 310, 311, 350, 352	81, 161, 175, 240, 246, 274, 310, 311, 350, 351	129, 191, 204, 240, 246, 304, 312, 350, 352, 354, 410, 468	240, 262, 278, 284, 300, 500, 522, 544, 608

results. Embryo biological assays are time-consuming and last for many days. Numerous repetitions of testing or performing other biological assays are also not always compatible with the routine. Fast and reliable assays would therefore be welcomed to assure the quality and stability of media used for embryo IVP. It is known that embryo culture conditions may have long-term effects on embryo, fetal and post-term development.[26,27]

For the analysis of embryo development percentages, we compared results from the same media batch on different experimental days, so that stability (or aging) could be followed. We also compared results from one aged batch (day 30 or day 45) with another fresh batch on the same experimental day. These two approaches were performed because it is known that IVP results may vary considerably when using bovine slaughterhouse-derived oocytes. Fertilized oocytes initiate the embryo development and may degenerate or present developmental block. Developmental block occurs in embryos from many species at specific embryo stages[28] and may be related to the cytoplasmic quality of the oocytes.[29] Bovine slaughterhouse-derived oocytes present extensive quality variation due to many factors, such as the season, the cow's breed, age and nutrition, or the time necessary for transportation to the laboratory.

Embryo development results indicate that, contrary to our previous expectations of a much reduced life time, media used for this work, stored for up to 45 days at +4°C, could be safely used in the IVP system. Conditions for preparation and storage may certainly influence media stability. We consider that, along with low temperature and stable pH, the maintenance of an atmosphere with 10% CO_2 and 90% N_2 inside the tubes contributed to prevent oxidation, which could speed medium degradation. In this way, stability of media may vary depending on the medium composition and conditions of storage.

Biological results observed in day 30 and day 45 media were confirmed by nano-ESI-MS fingerprinting. No significant variation among samples from different storage times could be noticed via PCA, indicating that chemical composition was not substantially affected by the storage period. Also, nano-ESI-MS fingerprinting coupled to PCA was able to differentiate the four media evaluated in this study.

Even though nano-ESI-MS fingerprints certainly do not detect all media components, since ion suppression plays a role, the sets of detectable ions have been found to function as proper markers for each media. We have employed the simplest sample-preparation-free protocol for nano-ESI-MS fingerprinting but, if needed, the use of some simple but selective extraction protocols may further improve the quality of discrimination between media.

For the biological assay from heat-shock media, even though no significant variation was observed, nano-ESI-MS fingerprinting and PCA from these media showed significant variation; hence, significant chemical changes occurred. For human oocytes, no obvious association between fertilization and cleavage and the quality of medium ascertained by the MEA could be observed.[30] Hence, a better way of evaluating the impact of changes in fingerprinting profiles in embryo development probably can be explored by identifying differential ions and measuring bovine embryo pregnancy rates. Such studies could identify critical substances in the media arising from thermal degradation or aging, without the need for biological assays in routine quality control.

In relation to the biological assay using frozen media, similar to the media exposed to heat shock, no significant variation in embryo production was noticed, but again the profiles from nano-ESI-MS fingerprinting were significantly affected. Based on differences in ion profile revealed by PCA, various freezing strategies could be evaluated by trying to match frozen media ESI-MS fingerprints to that of fresh control media, before introducing the use of biological assays.

Use of ESI-MS fingerprinting could also be expanded to detect reprotoxicity of reagents, consumables, and materials that are routinely used for *in vitro* production of the mammalian embryo. These evaluations are nowadays mainly based on biological assays.[31]

CONCLUSIONS

Direct infusion by nano-ESI-MS fingerprinting of bovine media was performed and compared to the conventional biological assay. After PCA, four different media (IVM, IVF, SOF and HSOF) were characterized and their profiles monitored as a function of storage time for up to 6 weeks with no signs of substantial degradation. Nano-ESI-MS profiles were significantly altered for media exposed to heat shock (+60°C for 3 h) and for those frozen at −70°C. Significant changes in chemical composition as detected by nano-ESI-MS were not accompanied by substantial decreases

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in embryo production. Although selectivity was already high enough for the crude samples, simple sample extraction protocols may be used to further increase selectivity of the chemical profiles or to evaluate specific classes of media components (e.g. peptides or lipids). Our results, using bovine embryo production as a model, indicate that nano-ESI-MS fingerprinting of the crude samples is suitable to evaluate embryo media chemical profiles after diverse storage conditions. The application of this technology should be optimized in each laboratory, depending on its specific culture medium and storage conditions and by the construction of a home-made database of ESI-MS fingerprints. We are currently evaluating nano-ESI-MS fingerprinting for monitoring embryo and fetal development and to determine the isolated effects of IVM, IVF and IVC media.

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