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Evaluation of different internal standardization approaches for the quantification of melatonin in cell culture samples by multiple heart-cutting two dimensional liquid chromatography tandem mass spectrometry

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ABSTRACT

We evaluate here different analytical strategies for the chromatographic separation and determination of N-acetyl-5-methoxytryptamine (MEL) and its oxidative metabolites N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), N1-acetyl-5-methoxykynuramine (AMK) and cyclic 3-hydroxymelatonin (c3OHM) in cell culture samples. Two dimensional liquid chromatography (2D-LC) in the multiple heart-cutting mode was compared with regular 1D chromatographic separations of MEL and its oxidative metabolites. Our results showed that the use of trifluoroacetic acid (TFA) as mobile phase modifier was required to obtain a satisfactory resolution and peak shapes particularly for c3OHM. As TFA is not compatible with ESI ionization the application of the MHC mode was mandatory for a proper chromatographic separation. We evaluate also different internal standardization approaches based on the combined use of a surrogate standard (5-methoxytryptophol) and an internal standard (6-methoxytryptamine) for MEL quantification in cell culture samples obtaining unsatisfactory results both by 1D- and 2D-LC-ESI-MS/MS (from 9 ± 2 to 186 ± 38%). We demonstrate that only the application of isotope dilution Mass Spectrometry through the use of an in house synthesized ^{13}C isotopically labelled analogue provided quantitative MEL recoveries both by using 1D- and 2D-LC-ESI-MS/MS (99±1 and 98±1. Respectively) in androgen-insensitive human prostate carcinoma PC3 cells.

1. Introduction

N-acetyl-5-methoxytryptamine, also known as melatonin (MEL), is produced enzymatically from the amino acid tryptophan. Although many tissues are capable of its production, MEL is the major night product of the pineal gland. Beside its role on the physiological adaptation to circadian rhythms, MEL displays powerful antioxidant and cytoprotective capabilities as well as neuroprotective and anti-cancer roles [1]. Its capability to decrease oxidative stress by removing free radicals is directly related to its concentration [2]. So, the highest amount of available antioxidant molecules, the highest capability to "buffer" the presence of free radicals that lead to oxidative damage and related diseases. To reduce oxidative damage, MEL initiates a cascade of reactions to produce several bioactive metabolites with excellent properties as free radical scavengers such as N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), N1-acetyl-5-methoxykynuramine (AMK) and cyclic 3-hydroxymelatonin (c3OHM) [3]. An accurate and precise quantifi-

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cation of MEL in cell culture samples is required to understand the molecular and cellular mechanisms involved in its neuroprotective and antitumor properties.

The monitoring of MEL in biological fluids has been typically performed by immunoassays as they provide a cost-effective analysis of large numbers of plasma/serum and saliva samples [4]. Radioimmunoassays (RIA) are very sensitive for the determination of MEL. They require a very low amount of sample, but they show the problem of handling and disposal of radioactive materials. Enzyme-linked immuno-sorbent assays (ELISA) are a good alternative to RIA, but both RIA and ELISA suffer from cross reactivity [5]. For example, cross reactivity to AMK and MEL was observed in a RIA based methodology developed to determine AFMK in plasma samples [6]. In addition, commercial inmunoassay kits have been reported to provide inaccurate daytime levels [7]. Fluorimetry has been also proposed for the determination of MEL but it shows low specificity too, as other endogenous substances may also generate or bind to fluorophores interfering the determination [7]. HPLC coupled to electrochemical, fluorimetric or UV detection have been used to detect MEL but due to the potential coelution of the analyte with electron donors, fluorophores or substances absorbing at the same wavelength as MEL, that may be present in the sample, an appropriate specificity cannot be assured. HPLC coupled to coulometric array detection has been applied to MEL determination in human plasma [8]. HPLC based methods have been applied as well for the determination of AFMK and AMK in neutrophil and peripheral blood mononuclear cell culture supernatants, with fluorimetric and UV–vis detection respectively showing acceptable results [9]. However, selectivity problems may arise affecting the results for certain sample matrices, and so requiring an efficient sample purification.

Selectivity problems can be overcome by using chromatographic techniques coupled to mass spectrometry (MS). Gas chromatography coupled to MS (GC–MS) has been applied to the determination of MEL showing good sensitivity and specificity [10]. However, MEL is not a volatile compound so a time-consuming derivatization step before GC–MS measurements is required. HPLC-MS is preferred over GC–MS as it allows a faster and an easier sample preparation while providing good sensitivity and high selectivity, especially when tandem MS instruments are used [11]. The main limitation of this technique is the availability of suitable internal standards, to correct for analyte losses during the sample preparation and for matrix effects during electrospray ionization [12,13]. Two-dimensional liquid chromatography (2D-LC) in the multiple heart-cutting (MHC) mode enables a purification of the sample while increasing the chromatographic resolution between analytes and interfering matrix compounds. In addition, this strategy is particularly useful when using mobile phases in the first dimension which are not compatible with the ESI source as demonstrated previously in our laboratory [14].

MEL has been determined in different biological fluids by HPLC-MS/MS using unlabeled internal standards [15] and deuterium-labeled internal standards [11]. MEL, AFMK and AMK have been quantified by HPLC-ESI-MS/MS in bovine follicular fluid and tissue culture medium using D_4–MEL [16]. Almeida et al. reported the synthesis of deuterated MEL and AFMK for human plasma analyses [17] and Hényková et al. [18] quantified MEL and AFMK in serum and cerebrospinal fluid using deuterated internal standards. Finally, Ma et al [19]. reported the determination of MEL, AFMK and AMK in mouse urine samples by HPLC-MS/MS and c3OHM by GC–MS using, for both approaches, 6-chloromelatonin as internal standard. Yet, there is a lack of reliable and fully validated analytical methods for the determination of MEL and its oxidative metabolites (c3OHM, AFMK and AMK).

In this work we evaluate different analytical strategies for the determination of MEL in cell culture samples by HPLC coupled to tandem MS. First, two dimensional liquid chromatography in the multiple heart-cutting (MHC) mode will be evaluated for the separation of MEL and its oxidative metabolites c3OHM, AFMK and AMK and compared with regular 1D separations.. Secondly, different internal standardization approaches will be evaluated and compared to isotope dilution mass spectrometry (IDMS) using an in-house synthesized ^{13}C labelled analogue to accurately quantify MEL in androgen-insensitive human prostate carcinoma PC3 cells.

2. Experimental

2.1. Reagents and materials

N-acetyl-5-methoxytryptamine (MEL), 6-methoxytryptamine and 5-methoxyindole-3-acetic were purchased from Sigma-Aldrich (St. Louis, MO, USA). N^1-acetyl-5-methoxykynuramine (AMK), N-[3-[2-(*formylamino*)−5-*methoxyphenyl*]−3-oxypropyl]-acetamide (AFMK) and 5-methoxytryptophol were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cyclic 3- hydroxymelatonin (c3OHM) and $^{13}C_1$-labelled melatonin were synthesized in the Laboratory of Natural Products Chemistry of the University of Warsaw (Poland). Acetonitrile (Optima ™ LC-MS Grade) was purchased from Fisher Scientific (Waltham, MA, USA). Trifluoroacetic acid (99%) and formic acid (>98%) were purchased from Sigma-Aldrich. Ammonia solution for analysis (EMSURE®, 28–30%) was purchased from Merck (Darmstadt, Germany). Ultra-pure water was produced by a Purelab Flex 3 water purification system from Elga Labwater (Lane End, UK).

2.2. Instrumentation

An Agilent 1290 Infinity 2D-LC system coupled to a to a triple quadrupole mass spectrometer Agilent 6460 equipped with an electrospray source with a jet stream was used throughout this work. The 2D-LC system was controlled by OpenLab CDS Chemstation and the triple quadrupole by MassHunter Acquisition software (Agilent Technologies). The first dimension incorporated a 1290 Infinity binary pump connected to an autosampler, thermostated column compartment, and a 1260 Infinity variable wavelength detector with a 10 mm flow cell. The two dimensions were interconnected by a 2-pos/4-port duo valve to which two distinct selector valves including six 40 or 80 µL sampling loops were coupled. The same system was used for conventional 1D-LC separations by connecting the 1D column directly to the MS system. A vortex mixer FB 15,024 (Fisher Scientific) was used for the homogenization of samples and working solutions. All solutions were prepared gravimetrically using an analytical balance model MS205DU (Mettler Toledo, Zurich, Switzerland).

2.3. Procedures

2.3.1. Synthesis of cyclic 3- hydroxymelatonin

The synthesis of c3OHM was based on a previous publication [20]. Briefly, 220 mg of Melatonin was dissolved in 200 mL of methylene chloride and methanol mixture (2:1, v/v). Then 1 mL of dry pyridine and 40 mg of Rose Bengal dye were added and the flask was immersed in ethanol/dry ice bath. Air was replaced by oxygen and the mixture was irradiated with 400 W halogen lamp for 10 h with vigorous stirring. Then the irradiation was terminated and oxygen was replaced by argon, 2 mL of dimethyl sulfide were added and the mixture was allowed to reach room temperature overnight. After evaporation under reduced pressure the residue was purified by column chromatography on alumina. Elution with chloroform allowed the recovery of unreacted melatonin (110 mg). Subsequent elution with 3% (v/v) methanol in methylene chloride yielded an amorphous solid of 70 mg of c3OHM.

2.3.2. Synthesis of $^{13}C_1$-labelled melatonin

A mixture of N-Acetyl-5-hydroxytryptamine (200 mg, 0,92 mmol), K_2CO_3 (381 mg, 2.76 mmol) and 18-Crown-6 (24 mg, 0.09 mmol) in 10 mL of acetone was stirred for 20 min at room temperature. Then, iodomethane-^{13}C (115µL, 1.84 mmol) was added and stirring at RT in closed sealing vial was continued for 5 days. After removing the solvent in vacuo, the residue was diluted with H_2O (50 mL) then extracted with chloroform (3 × 50 mL) and the combined organic phases were washed with brine (50 mL), dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography on silica gel (eluent: chloroform/MeOH 99:1) to obtain the target compound as white solid with 85% yield.

2.3.3. Cell culture

Androgen-insensitive human prostate carcinoma PC3 cells (Cat Number # CRL-1435TM) were obtained from "European Collection of Cell Cultures" (ECACC, Wiltshire, UK) and from "American Type Culture Collection" (ATCC, Rockville, MD). This androgen independent cell line is derived from an advanced bone metastasis. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma), 10 mM HEPES (Lonza, Basel, Switzerland), 2 mM L-glutamine (Lonza, Basel, Switzerland) and 1% antibiotics and antifungals (amphotericin B, penicillin and streptomycin) (Gibco, Grand Island, NY, USA). Cells were kept under controlled conditions in a CO2 incubator (New Brunswick ™ Galaxy®170 s, Eppendorf, Germany) at 37 °C and 5% CO2 atmosphere. For further analysis, cells treated and non-treated with melatonin were used. Cells were seeded in a Hyperflask (Corning, Ref #10,030) at a density of 106 cells/ml. Once reached the confluency, cells were collected using Trypsin 0,05% (Sigma) and seeded in another Hyperflask in order to obtain a sufficient cell substrate for further analysis. A cell pool from five replicates was produced to obtain a final homogenous and representative sample. The lyophilized pellet was finally homogenized so that different aliquots could be analyzed for recovery experiments. Treated and non-treated cells were seeded under the same conditions describe above. Before cells reached confluency, melatonin (1 M stock solution in 100% DMSO) at a final concentration of 1 mM was added. DMSO (final concentration of 0.1%) was added as vehicle to control cells. After 24 h, cells were washed three times with phosphate saline buffer (PBS), collected, centrifuged at 500 g for 10 min, washed again twice with PBS, recollected and frozen at −80 °C. Cell viability experiments were routinely performed with melatonin ranging from 1 nM to 1 mM. At these concentrations, melatonin exerts a decrease in cell proliferation without inducing cell damage and or cell death.

2.3.4. Sample preparation

The sample preparation for the analysis of the PC3 cell cultures was based on the application of a 3-cycle extraction procedure adapted from [21]. First, 20 mg of the frozen pellet were weighted in a microcentrifuge tube and a gravimetrically controlled amount of the surrogate internal standard 5-methoxytryptophol was added. Then the mixture was suspended in 500 µL of methanol cooled at −80 °C, snap-frozen in a liquid nitrogen/acetone bath and then thawed at room temperature. After vortexing for 30 s, the sample was centrifuged at 2000 g for 2 min and then the supernatant was transferred into a clean microcentrifuge tube. The pellet cells were suspended again in 500 µL of methanol at −80 °C and the freeze-thaw-vortex cycle was repeated. After centrifugation (2000 g, 2 min) the supernatant was transferred and pooled with the previous extract and the pellet cells were suspended in 250 µL of ultrapure water cooled at 5 °C to undergo the third freeze-thaw-vortex cycle. Then, the cells were pelleted by centrifugation (2000 g, 2 min) and the supernatant was transferred and pooled with the previous methanolic extracts. The pooled supernatant fractions were centrifuged 15,000 g for 2 min in order to remove cell debris. The supernatant was transferred to a fresh tube and evaporated to dryness using a centrifugal vacuum concentrator (37 °C). Finally, the dried extracts were reconstituted in 1250 µL of mobile phase and a gravimetrically controlled amount of the internal standard 6-methoxytryptamine was added to correct for measurement errors. When applying IDMS the surrogate internal standard 5-methoxytryptophol was replaced by $^{13}C_1$-labelled melatonin and no internal standard was added to correct measurement errors.

2.3.5. Chromatographic separation of the samples

The chromatographic separation by 1D-UPLC experiments was carried out by a reversed phase chromatography using a Zorbax RRHD Eclipse Plus C18 (3.0 × 50 mm, 1.8 µm, 95 Å pore size) column from Agilent. Ultrapure water with 0.1% formic acid, pH = 3.5 (A) and acetonitrile (B) were used as mobile phases and the flow rate was set at 0.4 mL min^{-1}. A volume of 5 µL was selected as injection volume for both, standards and samples and a gradient starting with 5.6% B for 3 min, from 5.6 to 22% of B until 8 min, from 22 to 26% B until 12 min and from 26 to 70 until 14 min was applied. In these experiments the effluent at the outlet of the column was directly sent to the mass spectrometer equipped with an ESI source.

The chromatographic separation by 2D-UPLC experiments was applied with the same injection volume, column, flow rate and gradient of the 1D experiments but using as mobile phases ultrapure water with 0.1% trifluoroacetic acid (TFA), pH = 2.2 (A) and acetonitrile with 0.1% TFA (B). The second dimension incorporated also a 1290 Infinity binary pump (Agilent Technologies) and a Zorbax Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm, 95 Å) from Agilent. Ultrapure water with 0.1% formic acid (A) and acetonitrile (B) at 0.4 mL min^{-1} were used as mobile phases in the second dimension. Taking into account the retention time of the target analytes and internal standards, fractions of 40 or 80 µL of the 1D mobile phase were stored in the sampling loops. Once the last compound was stored, they were transferred to the second dimension in reverse order. The chromatographic gradient of the second dimension started from 4% B to 80% B in 4 min.

2.3.6. Ionization and measurement of the samples by ESI-MS/MS

The ESI source working conditions were 3500 V as capillary voltage, 0 V as nozzle voltage, 30 psi as nebulizer pressure, 9 L min^{-1} as drying gas flow rate and 250 °C as drying gas temperature. The sheath gas flow rate and temperature were 12 L min^{-1} and 400 °C, respectively. The fragmentor voltage was set at 135 V. Table 1 shows the precursor ion, product ion and collision energy selected for the SRM measurements when quantifying the analytes by external calibration using 5-methoxytryptophol as surrogate internal standard and 6-methoxytryptamine as internal standard. When melatonin was quantified by IDMS using $^{13}C_1$-labelled melatonin, the isotopic distribution of the samples was measured by monitoring the transitions 233.1 →174.1, 234.1 → 175.1, 235.1 → 176.1 and 236.1 → 177.1 using a collision energy of 9 eV.

2.3.7. Calculation of melatonin concentration by IDMS and multiple linear regression

When applying IDMS and multiple linear regression with tandem MS the measured isotopic distribution (from i = 1 to i = n isotopologues) of a given fragment ion in the isotope-diluted sample $A_{mixture}$, can be assumed to be a linear combination of the isotopologue distribution of natural abundance fragment ion ($A_{natural}$) and that of the isotopically labelled fragment ion ($A_{labeled}$). The relative contribution of both isotope patterns in the experimental

Table 1
Precursor ion, product ion and collision energy selected for the SRM transitions of N-acetyl-5-methoxytryptamine (melatonin), N1-acetyl-5-methoxykynuramine (AMK), N-[3-[2-(*formylamino*)−5-*methoxyphenyl*]−3-oxypropyl]-acetamide (AFMK), cyclic 3- hydroxymelatonin (c3OHM) when quantifying the samples by external calibration using 6-methoxytryptamine as surrogate internal standard and 5-methoxytryptophol as internal standard.

Compound	Precursor ion	*m/z*	Product ion	*m/z*	CE (eV)
Melatonin	$C_{13}H_{17}N_2O_2$	233.1	$C_{11}H_{12}NO$	174.1	9
AFMK	$C_{13}H_{17}N_2O_4$	265.1	$C_{10}H_{12}NO_2$	178.1	11
c3OHM	$C_{13}H_{17}N_2O_3$	249.1	$C_{13}H_{15}N_2O_2$	231.1	7
AMK	$C_{12}H_{17}N_2O_3$	237.1	$C_{10}H_{12}NO_2$	178.1	7
5-methoxytryptophol	$C_{11}H_{14}NO_2$	192.1	$C_{11}H_{12}NO$	174.1	13
6-methoxytryptamine	$C_{11}H_{14}N_2O$	190.9	$C_{11}H_{12}NO$	174.1	9

mass spectrum are the molar fractions ($x_{natural}$) and ($x_{labeled}$) which can be calculated by solving Eq. (1):

$$\begin{bmatrix} A^1_{mixture} \\ \vdots \\ A^n_{mixture} \end{bmatrix} = \begin{bmatrix} A^1_{natural} & A^1_{labeled} \\ \vdots & \vdots \\ A^n_{natural} & A^n_{labeled} \end{bmatrix} \cdot \begin{bmatrix} x_{natural} \\ x_{labeled} \end{bmatrix} + \begin{bmatrix} e^1 \\ \vdots \\ e^n \end{bmatrix} \quad (1)$$

To apply this strategy, the isotopologue distribution of the natural and labelled fragment ions must be known in advance. They can be theoretically calculated knowing the fragmentation mechanism by suitable SRM dedicated software such as IsoPatrn© [22]. Then, molar fractions of analyte and labelled analogue can be calculated by multiple linear regression solving Eq. (1). The concentration of melatonin in the sample, $C_{natural}$, is then calculated by applying Eq. (2):

$$C_{natural} = C_{labeled} \cdot \frac{x_{natural}}{x_{labeled}} \cdot \frac{m_{labeled}}{m_{sample}} \cdot \frac{w_{natural}}{w_{labeled}} \quad (2)$$

Where $C_{labeled}$ is the concentration of $^{13}C_1$-labeled melatonin m_{sample}refers to the weight of the aliquot of sample analysed whereas $m_{labelled}$ refers to the weight of the labelled analogue solution added to the sample. $w_{natural}$ and $w_{labeled}$ refer to the molecular weights of natural abundance and labeled melatonin, respectively. Note that the labeled analogue must be previously characterized in terms of isotopic enrichment and purity for a successful application of Eq. (2) and thus avoiding calibration graphs [23].

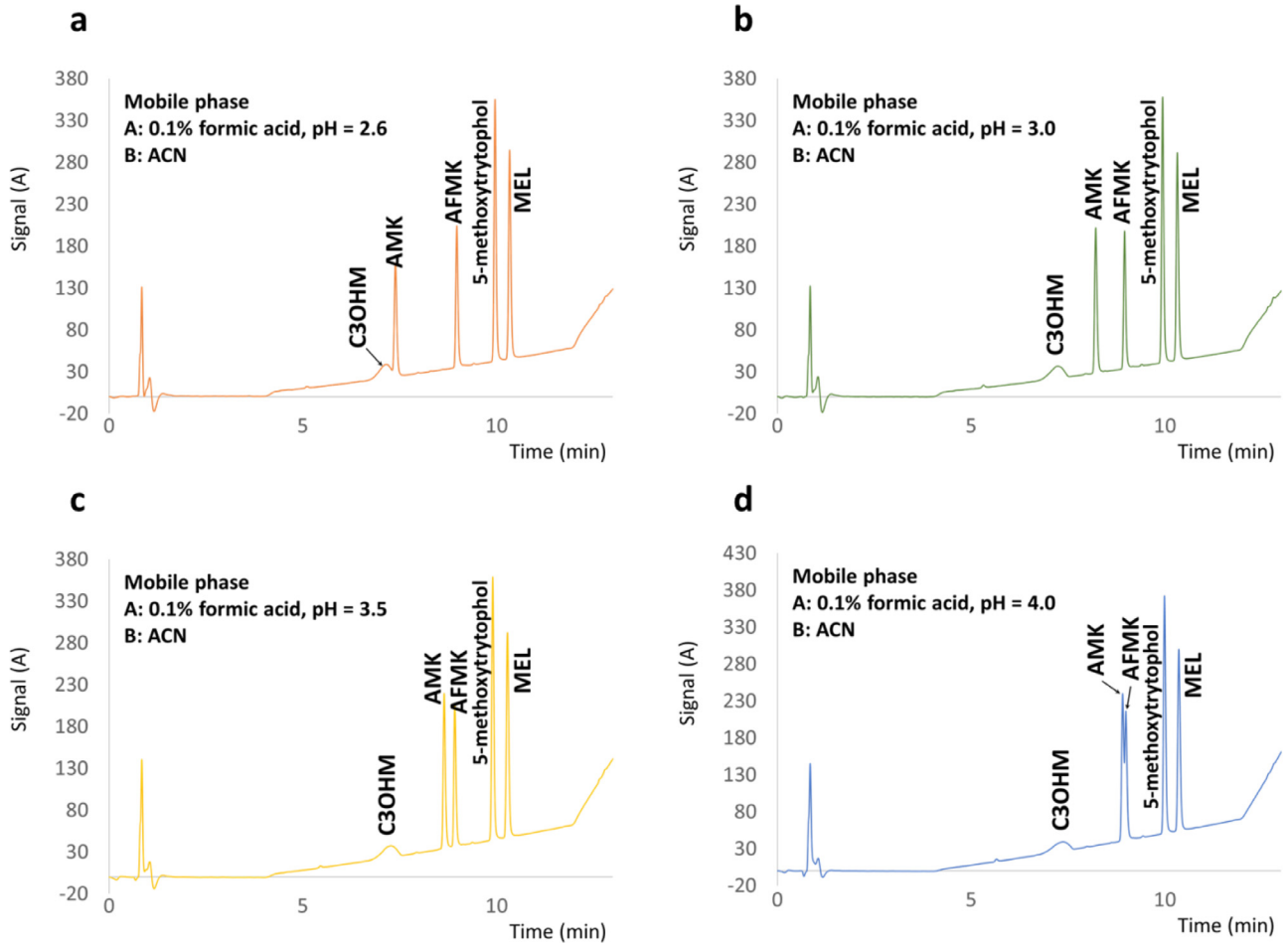

Fig. 1. 1D-LC-UV chromatograms of a standard containing 10 μg g^{-1} of MEL, c3OHM, AMK, AFMK and 5-methoxytrytophol using as mobile phase A ultrapure water with 0.1% FA at pH = 2.6 (a), pH =3.0 (b), pH = 3.5 (c) and pH = 4.0 (d) and ACN as mobile phase B with UV detection at λ = 231 nm.

3. Results and discussion

3.1. Optimization of the chromatographic separation of melatonin and its metabolites

A standard solution containing 10 μg g^{-1} of MEL, c3OHM, AMK, AFMK and the internal standard 5-methoxytrytophol were injected in the 1D-LC system with UV detection at λ= 231 nm for the optimization of the chromatographic separation. Mobile phases compatible with the ESI source (0.1% formic acid in ultrapure water and acetonitrile) were used at different pH values (2.6, 3.0, 3.5 and 4.0). Fig. 1 shows that the best chromatographic resolution for the four target analytes was obtained using pH=3.0 in less than 12 min under the optimized gradient summarized in Section 2.3. However, none of the tested pHs provided a good peak shape for c3OHM so the use of alternative mobile phase modifiers was considered.

Trifluoroacetic acid (TFA) increases the hydrophobicity of molecules by forming ion pairs with their charged groups enhancing the interaction of the molecules with the hydrophobic stationary phase and hence providing sharper and more symmetrical peaks [24]. Fig. 2 shows LC-UV chromatograms (λ= 231 nm) obtained using ultrapure water with 0.1% TFA and ACN as mobile phases. Fig. 2A shows the separation of MEL, c3OHM, AMK, AFMK and 5-methoxytrytophol. As expected, the use of TFA as mobile phase modifier improved the chromatographic separation and peak shape for all analytes and internal standards. Also it provided a lower background in UV detection and a shorter separation time. However, TFA is not suitable for ESI-MS measurements as it causes an important signal suppression [25]. In order to maintain the chromatographic resolution and peak shapes of the target compounds while avoiding ionization suppression effects A MHC 2D-LC strategy was applied as described previously [26]. 0.1% TFA in ultrapure water at pH = 2.2 (A) and acetonitrile with 0.1% TFA (B) were used as mobile phases on the first dimension. Then, 40 μL or 80 μL fractions taken at the analytes and the internal standards retention times are stored in sampling loops and transferred to the second dimension and measured by ESI-MS/MS in the SRM mode. The 2D separation was performed using a reverse phase column and ultrapure water with 0.1% formic acid (A) and acetonitrile (B) as mobile phases at 0.4 mL min^{-1} and using the chromatographic conditions summarized in Section 2.3. In this way, the 1D effluent was diluted and ionization suppression effects in the ESI source were minimized. The time windows of the 1D fractions were optimized before each measurement session injecting a standard solution containing the analytes and the internal standards into the LC-UV system.

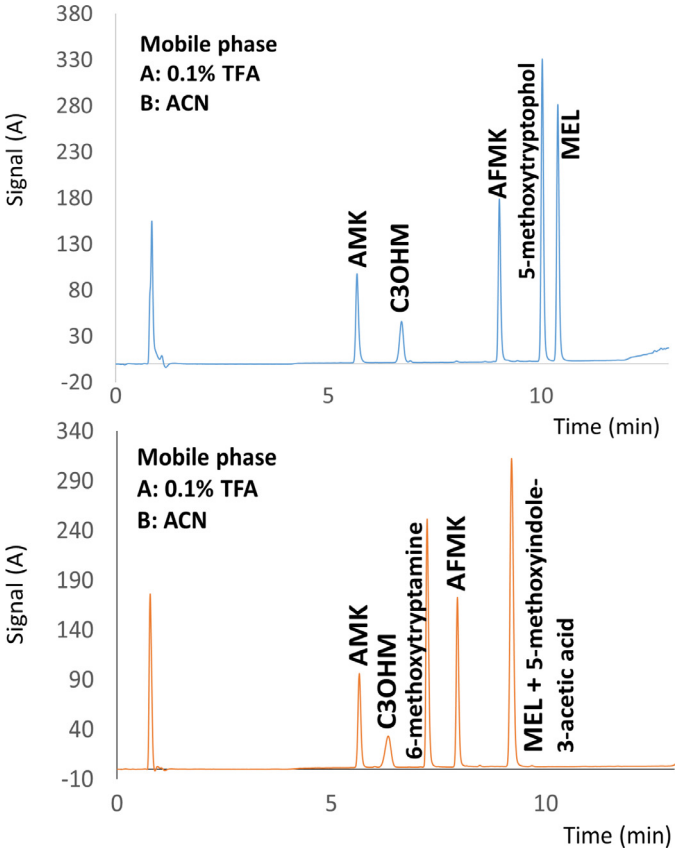

Fig. 2. 1D-LC-UV chromatograms (λ = 231 nm) obtained using ultrapure water with 0.1% TFA and ACN as mobile phases for A) MEL, c3OHM, AMK, AFMK and 5-methoxytrytophol and B) for MEL, c3OHM, AMK, AFMK, 6-methoxytryptamine and 5-methoxyindole-3-acetic acid.

3.2. Optimization of the instrumental settings for ESI-MS/MS detection in SRM mode

Scan measurements were performed first for all compounds to select the precursor ions. Figures S1A-S4A of the Supporting information show that the protonated molecular ion was the most intense for the four analytes and hence, it was selected as precursor ion. Product ion scans for each analyte are given in Figures S1B-S4B of the Supporting information and the optimized SRM transitions and collision energies are given in Table 1.

The ion source parameters were optimized to provide good sensitivity for the detection of the analytes. Figure S5 of the Supporting Information shows the variation of the SRM signals for MEL, c3OHM, AMK, AFMK at the different instrumental conditions tested. The four analytes showed a similar behavior for the optimized parameters so consensus values providing the highest signal for the four compounds were selected. The optimum values are indicated in Section 2.3.6.

3.3. Selection of internal standards

The determination of MEL and its metabolites in cell cultures requires several sample preparation steps that may cause undesired losses of the target compounds. Such losses will depend on the physicochemical properties of the compounds. Surrogate internal standards of similar chemical structure than the analytes are commonly added at the beginning of the sample preparation to correct for such errors. However, the chemical behavior of analyte and internal standards during sample preparation may be different affecting the analyte to internal standard ratio in the samples. In this work we evaluated the combined use of two internal standards, one to correct for incomplete recoveries through sample preparation (surrogate internal standard) and the other to correct for measurement variations (internal standard). N-acetyltryptamine has been previously used as internal standard for the determination of MEL in serum samples by ESI-MS/MS but unsatisfactory results were obtained [15]. Good accuracy and precision were obtained using 5-methoxytryptophol as internal standard for the determination of MEL in cell cultures by HPLC-UV [9]. Thus, 5-methoxytryptophol was tested as potential internal standard for the quantification of MEL and its oxidative metabolites by ESI-MS/MS. We also evaluated two additional compounds due to their similar chemical structure compared to MEL: 6-methoxytryptamine and 5-methoxyindole-3-acetic acid.

Fig. 2A shows that chromatographic resolution was achieved between the analytes and 5-methoxytryptophol using ultrapure water with 0.1% TFA and ACN as mobile phases allowing the application of 2D-LC in the MHC mode. Fig. 2B shows that 2,

5-methoxyindole-3-acetic acid coeluted with MEL, whereas 6-methoxytryptamine was baseline separated from the all the analytes. Thus, 2,5-methoxyindole-3-acetic was rejected as internal standard and instrumental settings for the ESI-MS/MS detection by SRM were optimized only for 5-methoxytryptophol and 6-methoxytryptamine. Scan and product ion scan measurements were performed to select the precursor and product ions, respectively as shown in Figure S6 and S7 of the Supporting Information. The absence of 6-methoxytryptamine and 5-methoxytryptophol was confirmed by injecting a cell culture extract in the 2D-UPLC-ESI-MS/MS.

3.4. Linearity assessment of the 1D- and 2D-LC-MS/MS calibration graphs

Taking into account the retention times of both internal standards, we decided to use 5-methoxytryptophol as internal standard to correct for sample preparation errors and 6-methoxytryptamine as internal standard to correct for ionization efficiency. Thus, 5-methoxytryptophol was added at the beginning of the sample preparation procedure and 6-methoxytryptamine after sample preparation but before injection in the LC system. First, a linearity assessment was carried out preparing calibration solutions of analyte concentrations between 0 and 1000 ng g^{-1} with a fixed 6-methoxytryptamine concentration of 50 ng g^{-1}. Fig. 3A shows a 2D-LC-ESI-MS/MS chromatogram of a standard solution containing MEL, c3OHM, AMK and AFMK (1000 ng g^{-1}) and the internal standards 5-methoxytryptophol and 6-methoxytryptamine obtained by 2D-LC-ESI-MS/MS system applying the MHC mode. Fig. 4 shows that the calibration curves obtained by 2D-LC-ESI-MS/MS were only linear for c3OHM but not for AMK, AFMK and MEL. These results were first tentatively ascribed to two potential effects derived from the use of the MHC strategy: i) occurrence of retention time shifts in 1D chromatographic peaks or ii) an incomplete transfer of the 1D peaks due to the limited volume of the storage loops (40 µL). The latter effect would become more pronounced at higher concentration levels due to the increase of the peak width.

In order to confirm this assumption, the same calibration graphs were measured by 1D-LC-MS/MS using mobile phases compatible with the ESI source (0.1% formic acid in ultrapure water and acetonitrile). As can be observed in Fig. 4 the same lack of linearity was observed for AMK, AFMK and MEL. Thus, loss of linearity was attributed to the ionization process [27] rather than to an incomplete transfer of the analytes into the second dimension or 1D retention time shifts. To overcome the linearity issue, samples were diluted when required, to ensure that they were measured within the linear range for each analyte: 0–400 ng g^{-1} for AFMK, 0–300 ng g^{-1} form AMK, 0–1000 ng g^{-1} for c3OHM and 0–100 ng g^{-1} for MEL.

3.5. Evaluation of different internal standardization approaches for melatonin quantification in cell culture samples

This study was carried out by analyzing fortified cell culture samples both by MHC-2D-LC-ESI-MS/MS and 1D-LC-ESI-MS/MS. We evaluated the use of a surrogate internal standard (surrogate IS) added at the beginning of the sample preparation procedure (5-methoxytrytophol), an internal standard (IS) added before the injection into the chromatograph and an isotopically labelled analogue $^{13}C_1$_MEL to apply isotope dilution mass spectrometry (IDMS). Recovery studies were carried out to evaluate the different internal standardization approaches. A homogenized PC3 cell culture of 250 mg was analyzed as described in Section 2.3.4. Three aliquots of 25 mg were analyzed per level of added concentration (0, 0.5 and 1.8 µg g^{-1}). Fig. 3B shows that only MEL is detected in the LC-MS/MS chromatogram of a non-fortified aliquot of the cell culture. This was expected as the cell culture was not subjected to stressing conditions able to initiate the melatonin antioxidant cascade. All cell culture samples were spiked with a gravimetrically controlled amount of the surrogate internal standard 5-methoxytrytophol at the beginning of the sample preparation (50 mg of a 1 µg g^{-1} solution) and a gravimetrically controlled amount of the internal standard 6-methoxytryptamine (50 mg of a 0.1 µg g-1 solution) before injection into the LC system. The samples were analyzed both by 1D-LC-MS/MS and MHC 2D-LC-MS/MS and recovery values were calculated plotting the added concentration vs the experimentally measured concentration. The experimentally measured concentration was calculated using four different strategies: 1) using the surrogate IS and the IS, 2) using only the surrogate IS, 3) using only the IS and 4) without using the surrogate IS nor the IS. Figure S8 of the Supporting Information shows the results obtained by 1D-LC-MS/MS and Figure S9 shows the results obtained by MHC 2D-LC-MS/MS. Table 2 summarizes the results obtained in all the experiments.

The endogenous concentration obtained under the different approaches was significantly different (from 0.35 to 7.39 µg g^{-1}) demonstrating the great influence of the internal standardization strategy. No statistical difference was found between the endogenous concentration obtained by 1D and 2D approaches. However, the uncertainty of the 2D values were about 2–3 times higher than that obtained by 1D. This was also the case for the uncertainty in the recovery values indicating reproducibility issues in the 2D strategy. Recovery values also depended on the internal standardization approach being the use of a surrogate IS the strategy leading to the best recovery (120 ± 8). A better linearity was also obtained by 1D in comparison with 2D. When analyzing the samples by MHC 2D-LC-MS/MS recovery values ranged from 19 to 186% with standard deviations up to 45% whereas the same samples analyzed by 1D-LC-ESI-MS/MS led to recoveries from 16 to 176% with standard deviations from 2 to 19%.

The recovery values obtained both by 1D and 2D approaches indicate that the use of 6-methoxytryptamine as IS leads to an overestimation of the experimental concentrations. This can be explained by the differences in the retention time between MEL and 6-methoxytryptamine that lead to a wrong correction of matrix effects in the ESI source. According to these results, the use of 6-methoxytryptamine as internal standard to correct from matrix effects in the quantification of MEL in cell culture samples is not recommended. In contrast, the use of 5-methoxytrytophol as surrogate IS provides better recovery values by 1D-LC-MS/MS than by the other approaches. According to the results obtained when no internal standardization is applied, the use of a proper surrogate IS is required as significant loses of sample are occurring during the sample preparation stage and/or MEL suffers from an important signal suppression due to matrix effects.

The unsatisfactory results obtained by MHC 2D-LC-MS/MS approaches can be ascribed to retention time shifts affecting the amount of analyte or internal standard transferred to the second dimension. Two strategies were follow to avoid the errors derived from the application of the MHC mode. The first was the use of higher volume loops that would allow the collection of the whole chromatographic peak regardless the occurrence of retention time shifts. The samples were reanalyzed storing the 1D fractions in 80 µL loops and monitoring the SRM transitions of MEL and 5-methoxytrytophol. As can be observed in Table 3 the accuracy and precision of the recovery values using 5-methoxytrytophol as surrogate IS improved from 56 ± 34% to 129 ± 10% when 80 µL loops were used. Unsatisfactory results were again obtained when no surrogate IS was used in the calculation of the concentration values. The second solution was the application of IDMS. In theory, using of an isotopically labelled analogue coeluting with the analyte, the same amount of analyte and labelled standard will be

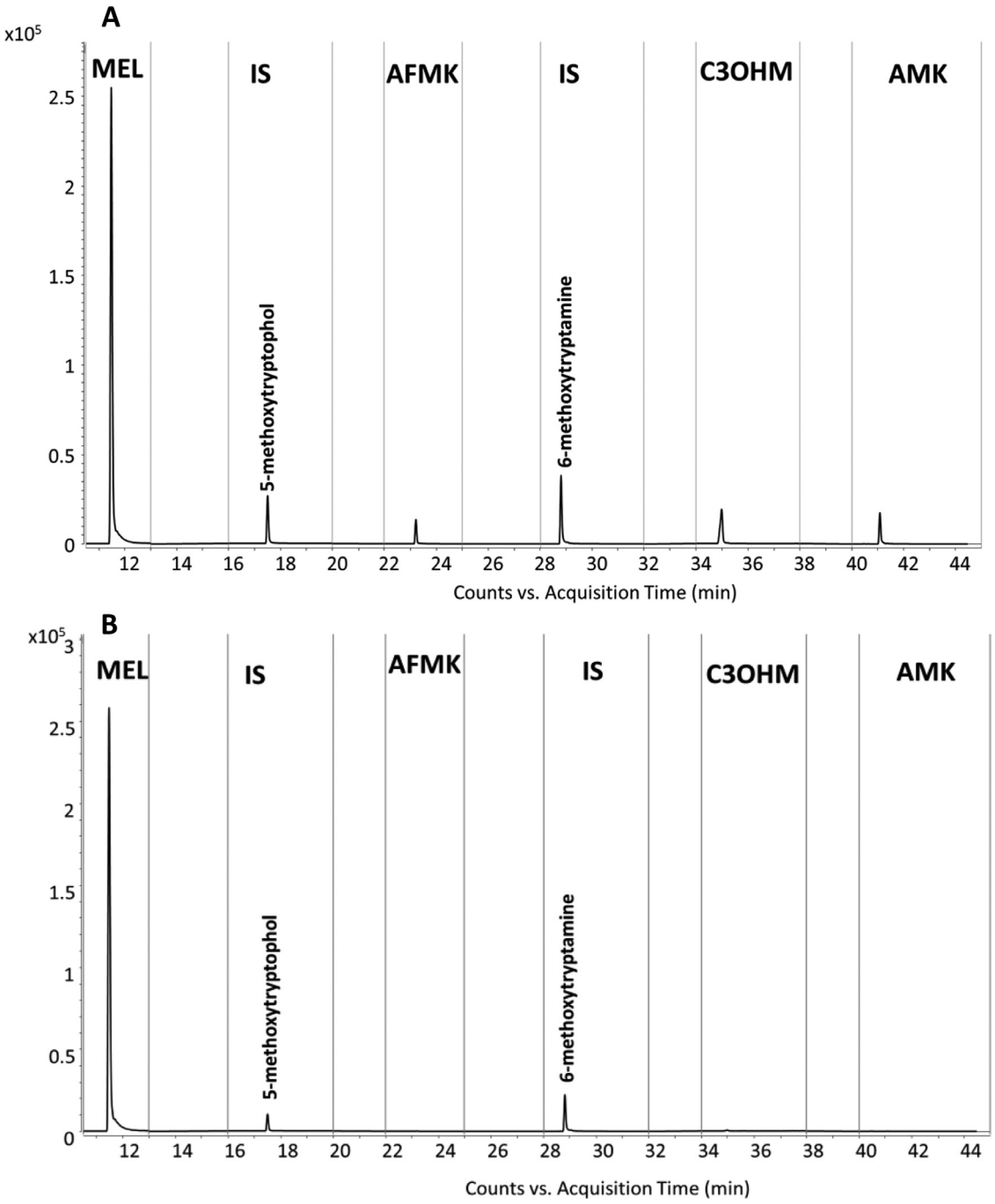

Fig. 3. 2D-LC-MHC-ESI-MS/MS chromatogram of a) a standard solution containing MEL, c3OHM, AMK and AFMK (1000 ng g^{-1}) and the internal standards b) a PC3 cell culture extract spiked with the internal standards.

transferred to the second dimension in each chromatographic run [26].

3.6. Quantification of melatonin in cell culture samples by isotope dilution mass spectrometry

3.6.1. Characterization of the in-house synthesized $^{13}C_1$_MEL

IDMS was applied to improve the accuracy and precision in the quantification of Melatonin in cell cultures both by 1D- and 2D-LC-MS/MS. Although commercially available, deuterated MEL analogues were not used to avoid isotope effects during sample preparation and chromatographic separation. Note that, for the successful application of the MHC mode, the coelution of analyte and its labelled analogue after chromatography is required which is not secured using deuterated standards. Therefore, we attempted the synthesis of $^{13}C_1$ labeled melatonin to minimize the occurrence of isotope effects particularly during the 1D chromatographic separation. The $^{13}C_1$_MEL was synthesized in collaboration with the Laboratory of Natural Products Chemistry at the University of Warsaw as and described in Section 2.3.2.

The successful application of IDMS using a multiple linear regression to avoid calibration graphs requires the previous knowledge of the isotopic enrichment and the purity of the labelled analogue [28]. The isotopic enrichment of the $^{13}C_1$-labelled MEL was calculated as described previously [29] obtaining an enrichment of 99.19 ± 0.01%. Then, the accuracy of the measurement of the iso-

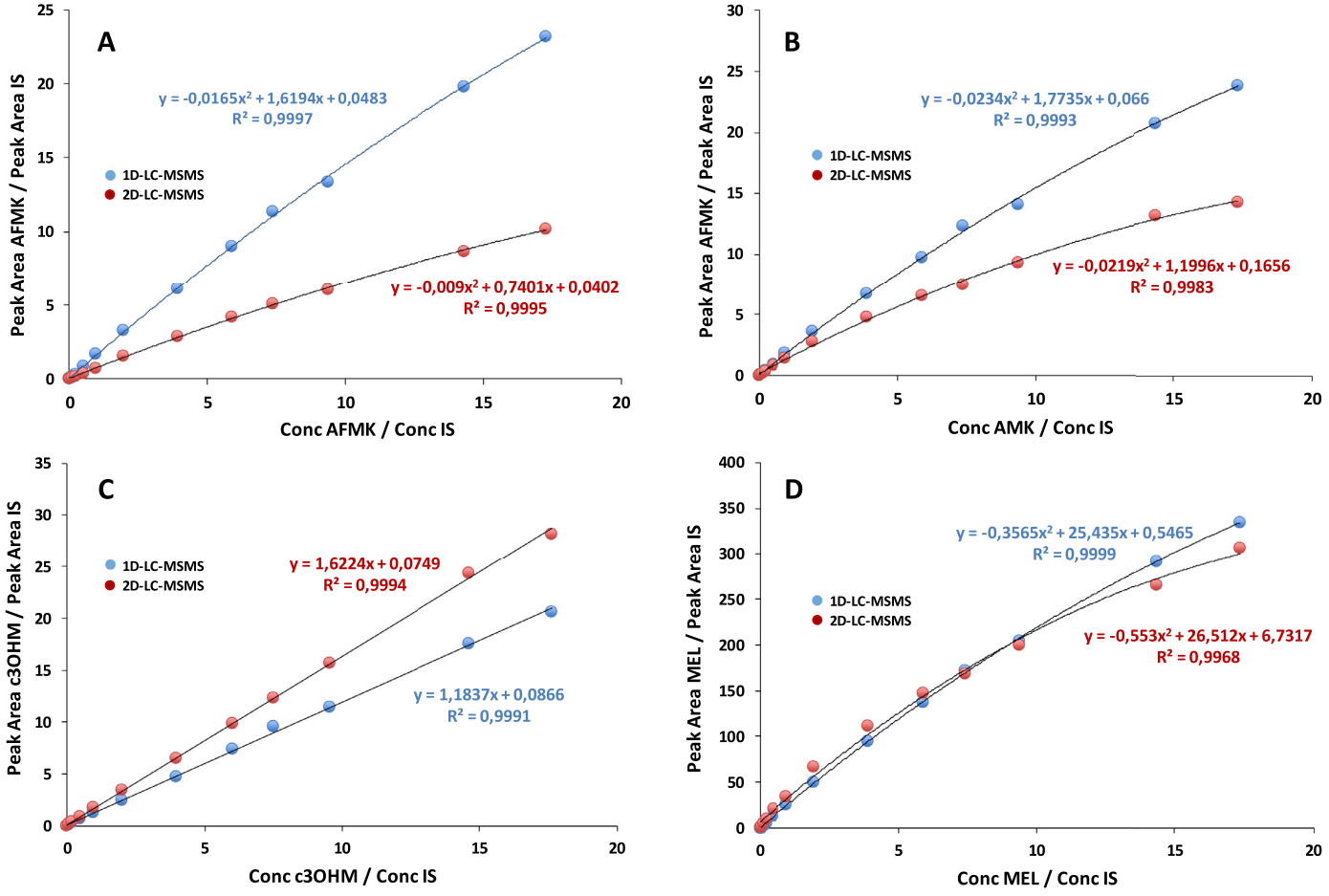

Fig. 4. Calibration curves for AFMK (A), AMK (B), c3OHM (C) and MEL (D).

Table 2
Slope x100 (%Recovery), intercept (endogenous concentration) and square of the correlation coefficient when plotting the added concentration vs the experimentally obtained concentration obtained for MEL in the cell culture extracts by 1D- or 2D-UPLC-ESI-MS/MS an internal standardization using 5-methoxitriptophol as surrogate internal standard (Surrogate IS) and 6-methoxytryptamine as internal standard (IS) (with 40 and 80 µL loops), as internal standard and using $^{13}C_1$-melatonin for IDMS quantification.

Sample	Added concentration (µg g^{-1})	Internal Standard-ization	Endogenous concentration (µg g^{-1}) (Intercept)			% Recovery (Slope x100)			Square of the correlation coefficient R^2		
			1D	2D (40 µL loop)	2D (80 µL loop)	1D	2D (40 µL loop)	2D (80 µL loop)	1D	2D (40 µL loop)	2D (80 µL loop)
Cell culture 1	0.5 and 1.8	Surrogate IS + IS	4.27 ± 0.17	4.74 ± 0.47		174 ± 17	93 ± 45		0.824	0.177	
		Surrogate IS	7.39 ± 0.08	7.00 ± 0.36	8.25 ± 0.11	120 ± 8	56 ± 34	129 ± 10	0.911	0.100	0.8624
		IS	2.81 ± 0.19	2.62 ± 0.41		176 ± 19	186 ± 38		0.783	0.505	
		None	0.35 ± 0.02	0.39 ± 0.03	0.33 ± 0.02	16 ± 2	19 ± 3	9 ± 2	0.811	0.683	0.474
Cell culture 2	0.3 and 2.7	Isotope Dilution	0.24 ± 0.01	0.25 ± 0.01		99 ± 1	98 ± 1		0.998	0.999	

topic distribution of the in-cell fragment ions measured by SRM for non-labeled and labeled MEL was studied. The experimental values obtained injecting MEL standards into the LC-MS/MS system were compared with the theoretical isotope distributions calculated by the SRM dedicated software IsoPatrn© [22]. Figure S10 shows the agreement between the theoretical and experimental isotope composition for natural abundance MEL and $^{13}C_1$_MEL. The SRM transitions selected to measure MEL concentration in cell cultures were 233.1→174.1, 234,1→175.1, 235,1→176.1 and 236.1→177.1. The concentration of the labeled standard was measured by reverse IDMS applying Eq. (1) and (2) after the analysis of blended solutions containing known amounts of labeled and a certified standard of natural abundance MEL. A concentration value of 1.067 ± 0.006 µg/g was obtained (Table S2) for the spike solution used in subsequent IDMS quantification experiments. Figure S11 shows the chromatograms obtained for a representative blend by 1D and 2D-LC-ESI-MS/MS. As can be observed, coelution of analyte and labelled analogue is observed under both chromatographic conditions. Therefore, potential errors derived from the transfer of the analyte from the 1D to the 2D are corrected as the same amounts of natural abundance MEL and $^{13}C_1$_MEL will be collected in the same fraction.

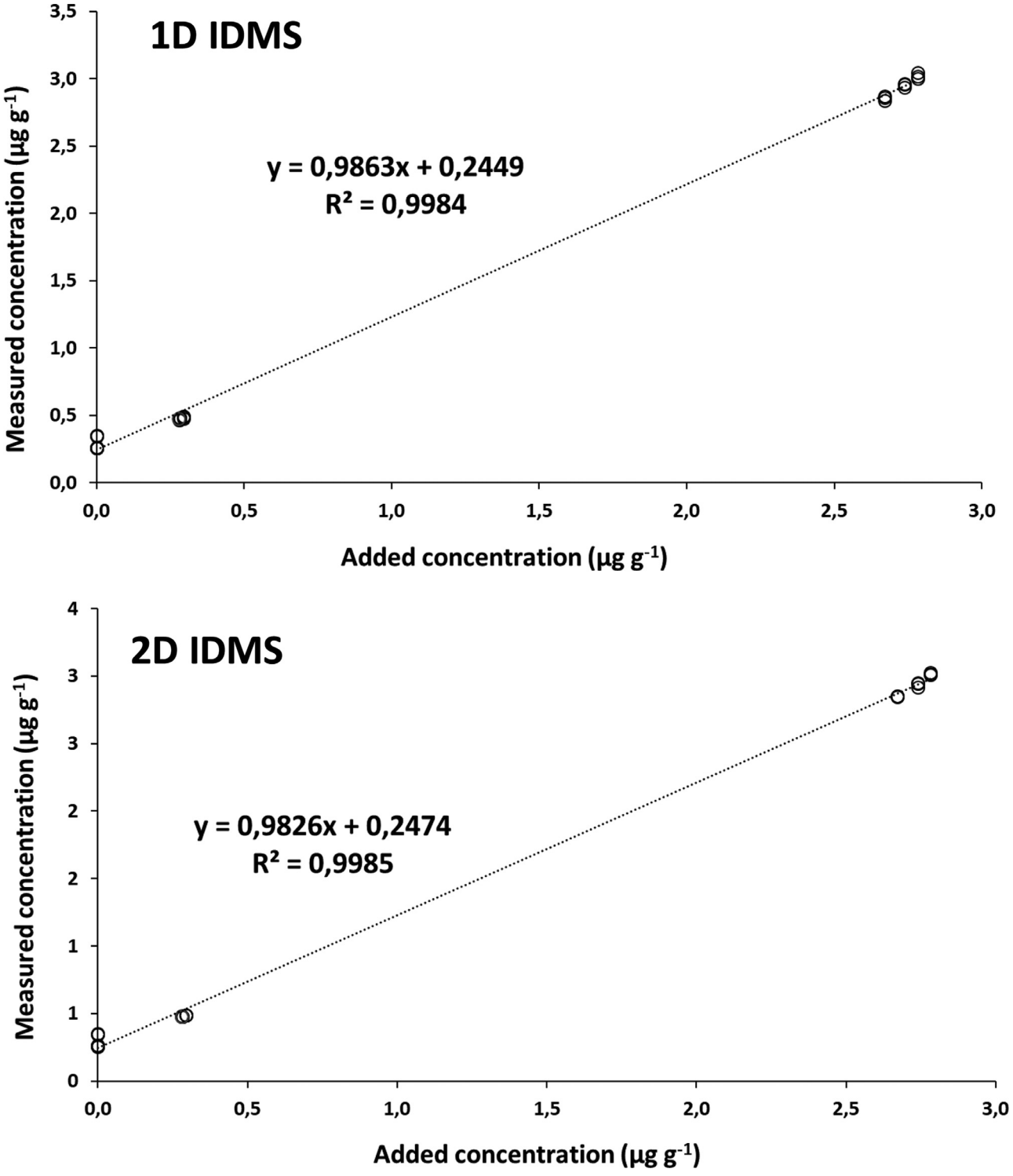

Fig. 5. Plot of measured versus added MEL concentration of a homogenized PC3 cell culture sample fortified with 0, 0.3 and 2.7 µg g^{-1}. The samples were measured by 1D-LC-MS/MS and 2D-LC-MS/MS and the MEL concentration was quantified by IDMS using $^{13}C_{1-}$MEL as labeled analogue. $n = 3$ independent replicates were performed for each concentration level and each replicate was injected in triplicate in the LC-MSMS. The points of the graphics correspond to individual injections in the LC-MSMS system.

3.6.2. Analysis of cell cultures samples

Recovery studies were carried out to evaluate the accuracy and precision of the IDMS strategy. Three aliquots of 25 mg of a second cell culture, containing a lower endogenous concentration of melatonin, were analyzed per level of added concentration (0, 0.3 and 2.7 µg g^{-1}) as described in Section 2.3.4. A gravimetrically controlled amount of $^{13}C_{1-}$MEL was added at the beginning of the sample preparation procedure to correct for analytical errors derived both from sample preparation and measurement. The MEL isotopic distribution in the samples were measured both by 1D- and 2D-LC-MS/MS. Recovery values were calculated plotting the added concentration vs the experimentally measured concentration. Fig. 5 shows the results obtained by 1D-LC-MS/MS and MHC 2D-LC-MS/MS while Table 2 summarizes the results obtained. As

can be observed, both approaches provide the same endogenous concentration in the cell culture: 0.24 ± 0.01 μg g^{-1} by 1D 1D-LC-MS/MS and 0.25± 0.01 μg g^{-1} by MHC 2D-LC-MS/MS. This is also the case for the recovery values 99±1 and 98±1, respectively. Also an excellent linearity when plotting the measured concentration vs the added concentration was obtained with both approaches. As expected, the IDMS quantification method provided much better accuracy and precision in comparison with the previous standardization approaches evaluated in this work, especially for 2D-LC-ESI-MS/MS.

4. Conclusions

An efficient chromatographic separation of MEL and its antioxidant metabolites c3OHMEL, AFMK and AMK was only possible using TFA as mobile phase modifier. As TFA is not recommended for ESI ionization, the application of this chromatographic conditions and MS detection is only enabled through the application of a 2D-LC strategy based in multiple heart cutting. 1D-LC-MS/MS using formic acid as mobile phase modifier did not provide satisfactory peak shape for c3OHMEL but enabled MEL separation from its metabolites. An efficient correction of the errors involved in the sample preparation and measurement of MEL in cell culture samples require the use of a proper internal standardization. This work demonstrated that only the application of IDMS through the use of an isotopically labelled analogue provided the required accuracy and precision compared to other internal standards eluting at different retention times. The results obtained for melatonin in this work suggest that the use of ^{13}C-labeled standards for the metabolites c3OHMEL, AFMK and AMK should provide precise and accurate 2D-LC-ESI-MS/MS quantification procedures for these compounds.

Author statement

Amanda Suárez Fernández, Adriana González Gago, Francisco Artime Naveda and Javier García Calleja carried out the analytical measurements. Anna Zawadzka and Zbigniew Czarnocki synthesized the $^{13}C_1$ labelled melatonin and the Cyclic 3-hydroxymelatonin. Juan Carlos Mayo Barrallo and Rosa M. Sainz Menéndez prepared the cell culture samples. Pablo Rodríguez-González, Adriana Goonzalez Gago and J. Ignacio García Alonso supervised the work and wrote the manuscript. Pablo Rodríguez González, J. Ignacio García Alonso, Juan Carlos Mayo Barrallo and Rosa M. Sainz acquired funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462752.

References

[1] A. Galano, D.X. Tan, R.J. Reiter, Melatonin as a natural ally against oxidative stress: a physicochemical examination, J. Pineal Res. 51 (2011) 1–16, doi:10.1111/j.1600-079X.2011.00916.x.
[2] R.T. Dauchy, E.M. Dauchy, J.P. Hanifin, et al., Effects of spectral transmittance through standard laboratory cages on circadian metabolism and physiology in nude rats, J. Am. Assoc. Lab. Anim. Sci. 52 (2013) 146–156.
[3] R.J. Reiter, J.C. Mayo, D.X. Tan, R.M. Sainz, M. Alatorre-Jimenez, L. Qin, Melatonin as an antioxidant: under promises but over delivers, J. Pineal Res. (2016) 253–278, doi:10.1111/jpi.12360.
[4] D.J. Kennaway, Measuring melatonin by immunoassay, J. Pineal Res. 69 (2020) e12657, doi:10.1111/jpi.12657.
[5] E.A. De Almeida, P. Di Mascio, T. Harumi, D.W. Spence, A. Moscovitch, R. Hardeland, D.P. Cardinali, G.M. Brown, S.R. Pandi-Perumal, Measurement of melatonin in body fluids: standards, protocols and procedures, Child's Nerv. Syst. 27 (2011) 879–891, doi:10.1007/s00381-010-1278-8.
[6] C. Harthé, D. Claudy, H. Déchaud, B. Vivien-Roels, P. Pévet, B. Claustrat, Radioimmunoassay of N-acetyl-N-formyl-5-methoxykynuramine (AFMK): a melatonin oxidative metabolite, Life Sci. 73 (2003) 1587–1597, doi:10.1016/S0024-3205(03)00483-1.
[7] D.J. Kennaway, A critical review of melatonin assays: past and present, J. Pineal Res. 67 (2019) e12572, doi:10.1111/jpi.12572.
[8] R. Condray, G.G. Dougherty, M.S. Keshavan, R.D. Reddy, G.L. Haas, D.M. Montrose, W.R. Matson, J. McEvoy, R. Kaddurah-Daouk, J.K. Yao, 3-Hydroxykynurenine and clinical symptoms in first-episode neuroleptic-naive patients with schizophrenia, Int. J. Neuropsychopharmacol. 14 (2011) 756–767.
[9] D. Hevia, J.C. Mayo, I. Quiros, C. Gomez-Cordoves, R.M. Sainz, Monitoring intracellular melatonin levels in human prostate normal and cancer cells by HPLC, Anal. Bioanal. Chem. 397 (2010) 1235–1244, doi:10.1007/s00216-010-3653-4.
[10] M.J. Paik, D.T. Nguyen, Y.J. Kim, J.Y. Shin, W. Shim, E.Y. Cho, J.H. Yoon, K.R. Kim, Y.S. Lee, N. Kim, S.W. Park, G. Lee, Y.H. Ahn, Simultaneous GC–MS analysis of melatonin and its Precursors as Ethoxycarbonyl/Pentafluoropropionyl derivatives in rat urine, Chromatographia 72 (2010) 1213–1217, doi:10.1365/s10337-010-1771-y.
[11] N. Özcan, Determination of melatonin in Cow's milk by liquid chromatography tandem mass spectrometry (LC-MS/MS), Food Anal.Methods 11 (2018) 703–708, doi:10.1007/s12161-017-1037-5.
[12] G.R. Martinez, E.A. Almeida, C.F. Klitzke, J. Onuki, F.M. Prado, M.H.G. Medeiros, P. Di Mascio, Measurement of melatonin and its metabolites: importance for the evaluation of their biological roles, Endocrine 27 (2005) 111–118, doi:10.1385/ENDO:27:2:111.
[13] K. Pape, C. Lüning, Quantification of melatonin in phototrophic organisms, J. Pineal Res. 41 (2006) 157–165, doi:10.1111/j.1600-079X.2006.00348.x.
[14] A. Suarez Fernandez, P. Rodríguez-Gonzalez, L. Alvarez, M. García, H. González Iglesias, J.I. García Alonso, Multiple heart-cutting two dimensional liquid chromatography and isotope dilution tandem mass spectrometry for the absolute quantification of proteins in human serum, Anal. Chim. Acta 1184 (2021) 339022.
[15] S. Yang, X. Zheng, Y. Xu, X. Zhou, Rapid determination of serum melatonin by ESI-MS-MS with direct sample injection, J. Pharm. Biomed. Anal. 30 (2002) 781–790, doi:10.1016/S0731-7085(02)00387-4.
[16] M.B. Coelho, M.C. Rodrigues-Cunha, C.R. Ferreira, E.C. Cabral, G.P. Nogueira, M.N. Eberlin, C.L.V. Leal, R.C. Simas, Assessing melatonin and its oxidative metabolites amounts in biological fluid and culture medium by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), Anal. Methods. 5 (2013) 6911–6918, doi:10.1039/c3ay41315b.
[17] E.A. Almeida, C.F. Klitzke, G.R. Martinez, M.H.G. Medeiros, P. Di Mascio, Synthesis of internal labeled standards of melatonin and its metabolite N1-acetyl-N2-formyl-5-methoxykynuramine for their quantification using an on-line liquid chromatography-electrospray tandem mass spectrometry system, J. Pineal Res. 36 (2004) 64–71, doi:10.1046/j.1600-079X.2003.00098.x.
[18] M. Hényková, E. Vránová, H.P. Amakorová, P. Pospíšil, T. Žukauskaite, A. Vlčková, M. Urbánek, L. Novák, O. Mareš, J. Kaňovský, P. Strnad, Stable isotope dilution ultra-high performance liquid chromatography – tandem mass spectrometry quantitative profiling of tryptophan-related neuroactive substances in human serum and cerebrospinal fluid, J. Chromatogr. A. 1437 (2016) 145–157, doi:10.1016/j.chroma.2016.02.009.
[19] X. Ma, J.R. Idle, K.W. Krausz, D.X. Tan, L. Ceraulo, F.J. Gonzalez, Urinary metabolites and antioxidant products of exogenous melatonin in the mouse, J. Pineal Res. 40 (2006) 343–349, doi:10.1111/j.1600-079X.2006.00321.x.
[20] A. Siwicka, R.J. Reiter, D.X. Tian, et al., The synthesis and the structure elucidation of N,O-diacetyl derivative of cyclic 3-hydroxymelatonin. Cent, Eur. J. Chem. 2 (2004) 425–433, doi:10.2478/BF02476198.
[21] C.A. Sellick, R. Hansen, G.M. Stephens, R. Goodacre, A.J. Dickson, Metabolite extraction from suspension-cultured mammalian cells for global metabolite profiling, Nat. Protoc. 6 (2011) 1241–1249, doi:10.1038/nprot.2011.366.
[22] L. Ramaley, L. Cubero Herrera, Software for the calculation of isotope patterns in tandem mass spctrometry, Rapid Commun. Mass Spectrom. 22 (2008) 2707–2714, doi:10.1002/rcm.3668.
[23] J.I. Garcia-Alonso, P. Rodriguez-Gonzalez, Isotope Dilution Mass Spectrometry, Royal Society of Chemistry, Cambridge (UK), 2013.
[24] M.C. García, The effect of the mobile phase additives on sensitivity in the analysis of peptides and proteins by high-performance liquid chromatography–electrospray mass spectrometry, J. Chromatogr. B 825 (2005) 111–123.

[25] T.M. Annesley, Ion Suppression in Mass Spectrometry, Clin. Chem. 49 (2003) 1041–1044.
[26] A. Suárez Fernández, P. Rodríguez-González, L. Alvarez, M. García, H. González Iglesias, J.I. García Alonso, Multiple heart-cutting two dimensional liquid chromatography and isotope dilution tandem mass spectrometry for the absolute quantification of proteins in human serum, Anal. Chim. Acta 1184 (2021) 339022, doi:10.1016/j.aca.2021.339022.
[27] C.M. Alfaro, A.O. Uwakweh, D.A. Todd, B.M. Ehrmann, N.B. Cech, Investigations of analyte-specific response saturation and dynamic range limitations in atmospheric pressure ionization mass spectrometry, Anal. Chem. 86 (2014) 10639–10645, doi:10.1021/ac502984a.
[28] P. Rodríguez-González, J.I. García Alonso. Mass Spectrometry-Isotope Dilution Mass Spectrometry. In Worsfold, P., Poole, C., Townshend, A., Miró, M., (Eds.), Encyclopedia of Analytical Science, (3rd ed.). vol. 6, pp 411–420, (2019) Elsevier. ISBN: 9780081019832.
[29] A. González-Antuña, P. Rodríguez-González, J.I. García Alonso, Determination of the enrichment of isotopically labelled molecules by mass spectrometry, J. Mass Spectrom. 49 (2014) 681–691, doi:10.1002/jms.3397.