Quantification of vitamin D3 and 25-hydroxyvitamin D_3 in food – The impact of eluent additives and labelled internal standards on matrix effects in LC-MS/MS analysis

Ložnjak Švarc, Petra; Barnkob, Line Lundbæk; Jakobsen, Jette

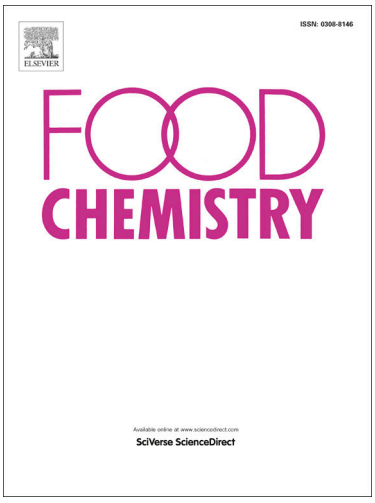
Published in:
Food Chemistry

Link to article, DOI:
10.1016/j.foodchem.2021.129588

Publication date:
2021

Document Version
Peer reviewed version

Journal Pre-proofs



Quantification of vitamin D_3 and 25-hydroxyvitamin D_3 in food – The impact of eluent additives and labelled internal standards on matrix effects in LC-MS/MS analysis

Petra Ložnjak Švarc, Line Lundbæk Barnkob, Jette Jakobsen

PII: S0308-8146(21)00594-X
DOI: https://doi.org/10.1016/j.foodchem.2021.129588
Reference: FOCH 129588

To appear in: *Food Chemistry*

Received Date: 6 November 2020
Revised Date: 28 February 2021
Accepted Date: 6 March 2021

Please cite this article as: Švarc, P.L., Barnkob, L.L., Jakobsen, J., Quantification of vitamin D_3 and 25-hydroxyvitamin D_3 in food – The impact of eluent additives and labelled internal standards on matrix effects in LC-MS/MS analysis, *Food Chemistry* (2021), doi: https://doi.org/10.1016/j.foodchem.2021.129588

Title

Quantification of vitamin D_3 and 25-hydroxyvitamin D_3 in food – The impact of eluent additives and labelled internal standards on matrix effects in LC-MS/MS analysis

Author names and affiliations

Petra LOŽNJAK ŠVARC[a]‡, Line Lundbæk BARNKOB[a]‡, Jette JAKOBSEN[a*]

[a] National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark

‡The authors contributed equally to this manuscript

[*]Corresponding author:

National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark, jeja@food.dtu.dk (J. Jakobsen)

Abstract

Deuterated vitamin D standards are used commonly as internal standards in LC-MS/MS analysis of vitamin D_3 and 25-hydroxyvitamin D_3 in food. However, the use of various eluent additives, such as methylamine, formic acid and ammonium formate, also contributes to matrix effects and the performance of analysis by affecting accuracy and robustness. For the first time, continuous post-column infusion experiments of isotopically labelled vitamin D_3-[d_6] were performed to evaluate ion-suppression in a wide variety of food (salmon, cheese, pork fat, pork meat, and egg yolk). Furthermore, results collected using five analytical methods, employing DAD/UV and MS/MS-detectors, were evaluated with in-house and standardised reference materials. The matrix effect was significant when analysing vitamin D_3 in most food matrices using the deuterium labelled internal standard. Even though the use of the $^{13}C_5$-labelled internal standard reduced matrix effects, a standardised method is needed to agree on the true value of vitamin D in food.

Keywords

electrospray ionization, mass spectrometry, post-column infusion, ion suppression, reference material, analogue

Chemical compounds studied in this article:

Vitamin D_3 (PubChem CID: 5280795), 25-hydroxyvitamin D_3 (PubChem CID: 5283731)

1. Introduction

Gaining knowledge about the contents of vitamin D vitamers, vitamin D_3 (vitD_3), 25-hydroxyvitamin D_3 (25(OH)D_3), vitamin D_2 (vitD_2), and 25-hydroxyvitamin D_2 (25(OH)D_2), in food is a challenge due to complicated analytical procedures. More than 40 years ago, use of vitD_2 as an internal standard for quantification of vitD_3, and vice versa, was introduced in high-performance liquid chromatography (HPLC) methods (Egaas & Lambertsen, 1979). Standard methods are now available for quantification of vitamin D in food, though they are based on the validation of a limited number of foodstuffs (CEN, 2009; ISO, 2018; NMKL, 2000). For 25(OH)D_3 and 25(OH)D_2, the use of an internal standard was introduced in 1995 (Mattila,

Piironen, Uusi-Rauva, & Koivistoinen, 1995), however, a standardised method has not yet been published. Through the years, development of chemical methods that need less sample amounts and are less work intensive have been continuous. HPLC-UV methods use 8-125 g of sample, extensive sample clean-up with liquid-liquid extraction (LLE) using vast amounts of solvents and preparative HPLC to provide a spectrum that shows a pure peak (Bilodeau et al., 2011; Byrdwell et al., 2013; Jakobsen & Saxholt, 2009). HPLC-UV methods are precise and accurate when a diode array detector (DAD) is used, but demand extensive lab work and have a high use of organic solvents (Jakobsen & Saxholt, 2009; Bilodeau et al., 2011). As liquid chromatography (LC) instruments coupled to a mass spectrometer (MS) became more common in research laboratories, methods using LC-MS with atmospheric pressure chemical ionization (APCI) for the analysis of vitamin D emerged (Dimartino, 2009; Heudi, Trisconi, & Blake, 2004; Strobel, Buddhadasa, Adorno, Stockham, & Greenfield, 2013). For the purpose of enhancing the sensitivity, avoiding interferences and thereby decreasing the sample amount and limits of detection (LOD) and quantification (LOQ), derivatisation methods using mainly 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) were introduced. PTAD increases the polarity of the molecule and thereby its ionization efficiency, making it possible to use electrospray ionization (ESI) (Barnkob, Petersen, Nielsen, & Jakobsen, 2019; Burild, Frandsen, Poulsen, & Jakobsen, 2014; Gomes, Shaw, Whitfield, & Hewavitharana, 2015; Lipkie et al., 2013). However, the use of ESI introduced a greater risk for matrix effects (ME) (Zhou, Yang, & Wang, 2017). In the analysis of vitamin D in food, numerous purification steps such as; saponification, LLE and a solid-phase extraction (SPE), are essential when using MS/MS-detection (Burild et al., 2014; Roseland et al., 2016). To ensure accurate and precise results and compensate ME, various structural analogues and isotopically labelled compounds are used as internal standards (Höller et al., 2010; Roseland et al., 2016; Trenerry, Plozza, Caridi, & Murphy, 2011).

Compounds with the same retention time, precursor and product ions are seldom the cause of interferences in LC-MS/MS. Interferences are caused by other co-eluting compounds that influence the ionization of the analyte (Bonfiglio, King, Olah, & Merkle, 1999). It is known that co-eluting matrix compounds may cause ion suppression or ion enhancement, although the underlying mechanism of ME is not yet fully elucidated (Matuszewski, Constanzer, & Chavez-Eng, 2003). It is generally assumed that stable isotope dilution assays (SIDA) using isotopically labelled internal standard will remove any relative ME, as it is presumed that it co-

elutes and behaves physically and chemically the same as the analyte of interest (Bonfiglio et al., 1999; Matuszewski, 2006; Matuszewski et al., 2003; Wu, Wiegand, LoRusso, & Li, 2013). However, deuterated internal standards are a little less lipophilic than the corresponding analyte and therefore elute slightly before the analyte. This difference in retention time can result in a different degree of ion suppression of the two analogues due to ME affecting their area ratio and thus the determined content (Wang, Cyronak, & Yang, 2007; Wieling, 2006). When using MRM (multiple reaction monitoring) or SRM (selected reaction monitoring) in MS/MS, only the peaks of interest are observed. However, undetected interferences can still have an impact on the signal. In the case of ion suppression, the LOD/LOQ will be increased, and if the internal standard and analyte do not co-elute, it can affect the recovery and thereby necessitate correction of the analytical result (Buhrman, Price, & Rudewicz, 1996). In some food, this was observed in our analyses for vitD_3, but not for 25(OH)D_3.

An overview of the causes, prevention, and applications of ion suppression has been published elsewhere, indicating that the eluent's gradient has to be optimized to ensure that the analyte and internal standard co-elute outside the ion suppression region if they do not have the same retention time (Furey, Moriarty, Bane, Kinsella, & Lehane, 2013; Trufelli, Palma, Famiglini, & Cappiello, 2011). Using recoverybased methods for detection of interferences does not give information on the chromatographic profile of the interference or of late-eluting interferences that, if not eluted, can affect subsequent runs (Bonfiglio et al., 1999). A full scan of a sample can sometimes give information about interfering compounds (Bonfiglio et al., 1999). The only method that also includes the chromatographic profile is the post-column infusion method, which was first described by Bonfiglio et al. (1999). The post-column infusion method requires a matrix blank, which is difficult to obtain when analysing nutrients in food. We assumed that disregarding the chromatographic separation, ME will affect the ionization of deuterated vitamin D_3 (vitD_3-[d_6]) and vitD_3 in the same manner; so we decided to use a continuous post-column infusion of vitD_3-[d_6] to evaluate ion suppression.

The aim of the study was to document that quantification of vitD in different food matrices may be based on UV- or MS/MS-detection, if the necessary clean up and internal standards are used. Firstly, we evaluate how the different additives (methylamine/formic acid, ammonium formate/formic acid and ammonium formate), and different internal standards (deuterated- and carbon-labelled vitD_3), affect ME in vitD_3 analysis in our in-

house reference materials (salmon, cheese, pork fat, pork meat, and egg yolk). Secondly, we compare results for vitD_3 and 25(OH)D_3 in food obtained by different methods, and evaluate which method provides the most accurate results by analysing three standardised reference materials (whole milk powder, meat homogenate, and whole egg powder).

2. Experimental

2.1. The rationale behind the study and its design

The analysis of in-house reference materials is part of the quality control performed to ensure satisfactory performance of the method, which we have performed accredited according to EN45001 since 1994 for vitD and according to ISO17025 since 2006 for 25(OH)D. The results have been collected over the years for UV- and MS/MS-methods used to analyse vitD_3 and 25(OH)D_3. In our first MS/MS-method we used methylamine as an additive (Burild et al., 2014), but due to instrument replacement, we had to omit methylamine as an additive. This resulted in a less robust method especially for meat, and for egg yolk, a dramatic change in recovery. To understand the reason for this observation, a post-column infusion of vitD_3-[d_6] was performed during the injection of reference materials usually used in vitD_3 analysis. The effect of eluent additives on the abundance of fragment ions obtained during LC-MS/MS analysis was tested using different ratios of ammonium formate and formic acid in the eluent. The experimental setup was exemplified on vitD_3. As a consequent of the results, vitD_3-[$^{13}C_5$] and 25(OH)D_3-[$^{13}C_5$] were introduced as internal standards. The results for the content of vitD_3 and 25(OH)D_3 in in-house reference and standardised reference materials were compared with former methods where vitD_2 and 25(OH)D_2 or vitD_3-[d_6] and 25(OH)D_3-[d_6] were used as internal standards, respectively.

2.2. Chemicals and reagents

All the chemicals used in the study were of analytical grade. The following chemicals were purchased from Sigma-Aldrich (Steinheim, Germany): vitD_3, vitD_2, 25(OH)D_3, 25(OH)D_2, petroleum ether, diethylether, anhydrous acetonitrile (99.8%), Fluka formic acid (LC-MS grade), methylamine, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and ammonium formate. Vitamin D_3-[26,26,26,27,27,27-d_6] and 25-hydroxyvitamin D_3-

[26,26,26,27,27,27-d_6] were purchased from Chemaphor Inc. (Ottawa, Canada), whereas vitamin D_3-[23,24,25,26,27-$^{13}C_5$] and 25-hydroxyvitamin D_3-[23,24,25,26,27-$^{13}C_5$] were purchased from Isosciences (Ambler, Pennsylvania). Ethyl acetate and 2-propanol were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). N-heptane and methanol (LC-MS grade) was from Honeywell (Steinheim, Germany), whereas ethanol (96%), sodium ascorbate and potassium hydroxide were purchased from Merck (Darmstadt, Germany). Ultra-pure Milli-Q water was made in-house (18.2 MΩ, Millipore, Bradford, USA).

2.3. Samples

Three NIST reference materials: NIST 1546a meat homogenate, NIST 1845a whole egg powder, and NIST 1549a whole milk powder (NIST, Gaithersburg, ML, USA), and seven in-house reference materials such as; salmon (2014), cheese (2014), pork fat (2010 and 2014), pork meat (2006), and pasteurized egg yolk (2012 and 2015) were used to quantify vitD_3 and 25(OH)D_3 by various methods. In post-column infusion experiments, NIST meat homogenate, and three additional meat samples were used (pork liver (2014), beef (2016), and pork meat (2016)). All in-house reference samples are bought in local supermarkets, homogenized, divided into portions of approximately 10 g and stored at -20 °C. Production year is indicated in parentheses.

2.4. Analytical methods

2.4.1. HPLC-UV-DAD method (UV-DAD)

A full description of the HPLC-UV-DAD method is publish elsewhere (Jakobsen, Clausen, Leth, & Ovesen, 2004; Jakobsen, Maribo, Bysted, Sommer, & Hels, 2007; Jakobsen & Saxholt, 2009). In short, 10-200 g of sample was saponified and extracted using LLE with petroleum ether:diethylether (300 mL in total) followed by silica SPE (2 g, 15 mL, ISOLUTE®, IST, Hengoed, UK), using 90 mL n-heptane/2-propanol in total. Final clean-up step was semi-preparative HPLC before the extract was injected into the HPLC-UV-DAD. Vitamin D was detected using DAD (220-320 nm) and quantified using UV (265 nm). Unlabelled standards of vitD_2 and 25(OH)D_2 were used as internal standards for quantification of vitD_3 and 25(OH)D_3, respectively.

2.4.2. LC-ESI-MS/MS method – methylamine/formic acid as eluent additives, vitD$_3$-[d$_6$]/25(OH)D$_3$-[d$_6$] as internal standards (MS-d$_6$-CH$_3$NH$_2$/HCOOH)

The method is published elsewhere (Burild et al., 2014). Briefly, 1 gram of sample was saponified, extracted by LLE using ethyl acetate:n-heptane (total 30 mL), followed by silica SPE (ISOLUTE®, IST, Hengoed, UK), where 12 mL n-heptane/2-propanol in total was used. The sample was derivatised using PTAD before injection on HPLC-MS/MS. The system used for quantification of vitamin D vitamers was Agilent 1200 series HPLC coupled to Agilent 6460 series Triple Quadrupole Mass Spectrometer (MS) (Agilent Technologies, Santa Clara, CA). Eluent A consisted of Milli-Q-water, 5 mM methylamine and 0.1% formic acid, whereas eluent B contained methanol (MeOH) with 5 mM methylamine and 0.1% formic acid. The methylamine adducts were used as precursor ions, whereas product ion with *m/z* 298.0 was used for quantification in following MRM transitions: *m/z* 607.4 → 298.0 for 25(OH)D$_3$, *m/z* 591.4 → 298.0 for vitD$_3$, and *m/z* 613.4 → 298.0 for 25(OH)D$_3$-[d$_6$] and *m/z* 597.4 → 298.0 for vitD$_3$-[d$_6$] as their corresponding internal standards.

2.4.3. LC-ESI-MS/MS – ammonium formate as eluent additives, vitD$_3$-[d$_6$]/25(OH)D$_3$-[d$_6$] as internal standards (MS-d$_6$-HCOONH$_4$)

A full description of the method is published elsewhere (Barnkob et al., 2019). In short, 1 gram of sample was saponified, extracted by LLE using ethyl acetate:n-heptane (total 30 mL), followed by Hybrid SPE (30 mg/1 mL column, Supelco Analytical, Bellefonte, PA, USA), where 1,5 mL of 0.1% acetonitrile in formic acid in total was used. The sample was derivatised using PTAD before injection on HPLC-MS/MS. In the meantime, we received a new 6470 series Triple Quadrupole MS (Agilent Technologies, Santa Clara, CA). The mobile phase system was composed of eluent A: Milli-Q-water:methanol (95:5) with 2.5 mM ammonium formate and eluent B: methanol with 2.5 mM ammonium formate. The protonated adduct was used as precursor ion and the product ion with m/z 298.1 was used for quantification of vitD$_3$ and 25(OH)D$_3$ using following MRM transitions: *m/z* 576.3 → 298.1 for 25(OH)D$_3$, *m/z* 560.3 → 298.1 for vitD$_3$, and *m/z* 582.3 → 298.1 for 25(OH)D$_3$-[d$_6$] and *m/z* 566.3 → 298.1 for vitD$_3$-[d$_6$] as their corresponding internal standards. The same method was used for determining the effect that eluent additives have on adduct formation where 0.05% formic

acid was added as eluent additive to both eluents (MS-d_6-$HCOONH_4$/HCOOH). Furthermore, we extended the runtime to 18 min (Supplementary Material (SM), Table S1), thus having a longer equilibration time, while keeping the same retention time for $vitD_3$.

2.4.4. LC-ESI-MS/MS – ammonium formate as eluent additive, $vitD_3$-[$^{13}C_5$]/$25(OH)D_3$-[$^{13}C_5$] as internal standards (MS-$^{13}C_5$-$HCOONH_4$)

The effect of the use of $^{13}C_5$-labelled internal standards was also tested using the method from section 2.4.3. Both eluents contained 2.5 mM ammonium formate as eluent additive, and the following MRM transitions were used for quantification: *m/z* 576.3 → 298.1 for $25(OH)D_3$, *m/z* 560.3 → 298.0 for $vitD_3$, and *m/z* 581.3 → 298.1 for $25(OH)D_3$-[$^{13}C_5$] and *m/z* 565.3→ 298.1 for $vitD_3$-[$^{13}C_5$] as their corresponding internal standards.

2.5. Post-column infusion

2.5.1. Preparation of sample matrix

The samples described in section 2.3. and blank (water) were prepared according to the sample clean-up described in section 2.4.3. The extracts were stored at -20 °C until analyses.

2.5.2. Derivatised $vitD_3$-[d_6] solution for continuous post-column infusion

50 µL of the 50 µg/mL solution of deuterium-labelled $vitD_3$-[d_6] was evaporated to dryness under nitrogen and re-dissolved in 2 mL of 0.3 mg/mL PTAD in anhydrous acetonitrile. The mixture was shaken and left to derivatise in the darkness for 5 min, followed by the addition of 0.6 mL of Milli-Q water. The solution was transferred to a 50 mL volumetric flask and filled with methanol. The final concentration used for continuous post-column injection was 50 ng/mL.

2.5.3. LC-MS/MS conditions during post-column infusion

Post-column infusion experiments were performed using the methods described in 2.4.2 and 2.4.3 sections. The effect of eluent additives was particularly evaluated in MS-d_6-$HCOONH_4$ method (2.4.3), where besides ammonium formate, the addition of 0.05% formic acid to mobile phases was evaluated. Gradient runs were used in all post-column infusion experiments (SM, Table S2).

Quantification was performed on an Agilent 6470 Triple Quadrupole MS equipped with a Jet Stream ion source (Agilent Technologies, Santa Clara, CA, USA) using positive multi reaction monitoring (MRM) mode. The

specific instrument parameters can be found elsewhere (Barnkob et al., 2019). The $[M+H]^+$ ions of derivatised analytes used as precursor ions for $vitD_3$ and $vitD_3$-$[d_6]$ were as described in 2.4.2 and 2.4.3 when methylamine and ammonium formate/formic acid were used, respectively. *m/z* 298.1 was used as a product ion for all the analogues. In addition, a product ion scan method using a collision energy of 10 and scanning from *m/z* 150-600, was set up for the chosen precursors: $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$ and $[M+H+NH_4]^{2+}$ at *m/z* 560.3, 582.3, 577.3 and 289.2, respectively, to examine the eluent effect on abundance using MS-d_6-$HCOONH_4$ method.

4 µl/min of $vitD_3$-$[d_6]$ was infused post-column through a tee using a KD Scientific syringe pump (Holliston, MA, USA) with a 1 mL SGE gastight syringe during the chromatographic runs. The whole sequence was run twice, once with 0% formic acid in the eluents, and then with 0.05% formic acid in the eluents. In both cases, a run without injection was included. The infusion chromatograms were visualized using Agilent MassHunter Qualitative Analysis (v. B.07.00). Chromatograms were overlaid regarding the retention time of inherent $vitD_3$ in the samples if there were small shifts in retention time between runs.

2.6. Matrix effect studies

ME were examined by preparing different food extracts, as described in section 2.4.3., but without the addition of internal standards. Following the extraction procedure, the extracts of in-house reference materials of salmon, cheese, pork fat, egg yolk, beef and blank (water), were reconstituted in acetonitrile containing 0.1% formic acid. The reconstituted extract (325 µL) was added to the activated Hybrid-SPE and the purified extract is collected in the glass tube. The same amounts of labelled and unlabelled internal standards (8 ng) were added to three SPE-purified extracts for each of the examined food matrices. Nothing was added to one of the SPE-purified extracts to analyse the response of endogenous vitamin D vitamers in these foods. All SPE-purified extracts were derivatised using PTAD and analysed by LC-MS/MS. The percentage of matrix effect (%ME) on the analysis of each vitamin D vitamer was calculated by the following equation (Matuszewski et al., 2003):

$$\%ME = \frac{(\text{Area in food matrix with 8 ng standard} - \text{Area in food matrix without standard})}{(\text{Area in water matrix with 8 ng standard} - \text{Area in water matrix without standard})}$$

Relative matrix effect (%RME) was calculated as percentage of the ratio between %ME of the analysed vitamin D vitamer and the %ME of the corresponding internal standard used for the quantification of the analyte of interest.

2.7. Statistical analysis

All results for $vitD_3$ and $25(OH)D_3$ contents are given as the mean ± standard deviation (SD) of independent determinations when $n \geq 3$. One-way analysis of variance (ANOVA) was used to evaluate significant differences in the $vitD_3$ and $25(OH)D_3$ content within the same in-house and standardised reference materials analysed by different analytical methods. Tukey's post hoc test was used to determine a significant difference between analytical methods where p-values <0.05 were classified as significantly different. Statistical analyses were performed using JMP® Statistical Discovery software version Pro 15.0.0 (SAS Institute Inc. Cary, NC, USA).

3. Results and discussion

3.1. The effect of eluent additives on the ion suppression

The replacement of the eluent additive of methylamine/formic acid to ammonium formate, meant that it was necessary e.g. for egg yolk, to correct for recovery, which was up to 135%. If not corrected for recovery, the content of $vitD_3$ in egg yolk would be overestimated. In our runs of meat matrices we randomly observed lower than 100% recovery. However, for other matrices and for $25(OH)D_3$, the recovery was varying, but in general it was approx. 100%. As mentioned, $vitD_3$-[d_6] was used as an internal standard, and although it elutes 0.019 minutes before $vitD_3$, we expected the ion suppression to be comparable to $vitD_3$. We hypothesized that ME was the primary cause of our observation, which we evaluated by using the continuous post-column infusion method. To our knowledge, we are the first to report that post-column infusion experiments can be performed by using isotopically labelled internal standards when a blank matrix is not available. Although there might be a small difference in how ME affect isotopically labelled internal standards and their analogues, it is the closest we found to the physical and chemical likeness of the analyte (Berg & Strand, 2011).

Ion suppression chromatograms obtained from running sample extracts of salmon, cheese, egg yolk, and liver gave different ion suppression patterns. $VitD_3$ elutes at approximately 6.3 minutes, and differences in ion suppression were observed between 5 and 7 minutes (Figure 1). For salmon, cheese, and egg yolk extracts, an

addition of 0.05% formic acid to 2.5 mM ammonium formate (pink in Figure 1) decreased the ion suppression compared with ammonium formate alone (blue in Figure 1). However, in the liver matrix, no difference was found in ionization efficiency when using different eluents, and the ion suppression was almost completely stable during the elution of the $vitD_3$ analogous (Liver in Figure 1).

Figure 1. Post column infusion of $vitD_3$-[d_6] (*m/z* 566.3 → 298.1) obtained while running the extracts of salmon, cheese, egg yolk and liver. A segmented linear gradient with 75% MeOH to 100% (2.5.3) was used. The chromatograms denoted with sample names are the full chromatograms, while the chromatograms below each one are zoomed-in. The vertical line in the full chromatograms shows the retention time of $vitD_3$ while zoom-ins are overlaid with the $vitD_3$ peak (*m/z* 560.3 → 298.1). The pink was obtained using an eluent with 2.5 mM ammonium formate, and blue using an eluent with 2.5 mM ammonium formate + 0.05% formic acid.

The ion suppression chromatograms obtained from injection of extracts of various meat samples (meat homogenate, pork meat and beef) were comparable when 2.5 mM ammonium formate was used alone, and more or less identical when a combination of 2.5 mM ammonium formate and 0.05% formic acid was used. (Figure 2).

Figure 2. Post-column infusion chromatograms of $vitD_3$-[D_6] (*m/z* 566.3 → 298.1) during injection of extracts of meat homogenate (purple), pork meat (2006, orange), pork meat (2016, black), beef (pink) and pork fat (brown) using 2.5 mM ammonium formate (left) or 2.5 mM ammonium formate + 0.05% formic acid (right) as the eluent additive (the run of pork fat failed and was therefore omitted from 2.5 mM ammonium formate alone). A segmented linear gradient with 75% MeOH to 100% MeOH was used (2.5.3). The vertical line in the upper chromatograms shows the retention time of $vitD_3$ while the lower chromatograms are overlaid with the $vitD_3$ peak (*m/z* 560.3 → 298.1). Above is the full chromatogram, below is a zoom-in on the $vitD_3$ peak.

To evaluate the effect of methylamine (combined with formic acid) on the ion suppression, extracts of blank, egg yolk, meat homogenate, and pork meat were analysed. At the retention time of $vitD_3$, no ion suppression was visible in the infusion chromatogram of the blank extract (SM, Figure S3). As for the blank extract, less

ME in the egg yolk, meat homogenate, and pork meat extracts were observed (Figure 3). This indicated that methylamine adduct of vitD_3-[d_6] ([M+CH_3NH_3]$^+$, *m/z* 597.4 → 298.1) was much less prone to ion suppression.

Figure 3. Post-column infusion chromatograms of the methylamine adduct of vitD_3-[D_6] ([M+CH_3NH_3]$^+$, *m/z*597.4 → 298.1) obtained during run of extracts of egg yolk and meat homogenate (purple) and pork meat (2006, orange). The eluent contained 5 mM methylamine and 0.1% formic acid combined with a segmented linear gradient with 60% to 100% MeOH (2.5.3).

We exploredthe physical differences between the additives methylamine/formic acid and ammonium formate. The solution of 5 mM methylamine and 0.1% formic acid in water had a pH of 3.2, while the solution of 2.5 mM ammonium formate in water had a pH of 6.1. Formic acid has a pKa of 3.75, and ammonium has a pKa of 9.25. Thus, ammonium formate will not function as a buffer at pH 6.1 (Konermann, 2017)**.** Adding formic acid (0.05%) to ammonium formate in water lowered the pH to 3.2, which was within the buffering capacity. During ESI, the water is oxidized, whereby the pH can be lowered by up to 4 pH units. However, the pH is mostly affected by systems that are not buffered and around neutral pH (Van Berkel, Zhou, & Aronson, 1997). An increase in the ionization efficiency with an increased percentage of methanol (75% to 100%) in the eluent was observed in the post-column infusion chromatogram obtained without injection (SM, Figure S4). Furthermore, the combination of 2.5 mM ammonium formate and 0.05% formic acid as eluent additives decreased the ionization efficiency and delayed the effect of increasing the percentage of methanol compared with an eluent with 2.5 mM ammonium formate alone, resulting in the elution of the vitD_3 analogues in an area with stable ionization efficiency in regard to the eluent (SM, Figure S4). This shows the effect of the mobile phase composition, which has been reported previously (Dams, Benijts, Günther, Lambert, & De Leenheer, 2002). Based on the information from the post-infusion experiment, the use of methylamine as an additive would benefit vitamin D_3 analysis, but we observed an unacceptable decrease of ~50% in the signal in vitamin K analysis. Furthermore, we observed that the abundances of vitD_3 when using ammonium formate

were a factor 10 higher than for methylamine for extracts of food (Figure 1, 2, 3) as well as for blank (SM, Figure S3 and S4).

3.2. The effect of ammonium formate and formic acid on the abundance

As mentioned above, theddition of 0.05% of formic acid to the eluents stabilized the ion suppression during the elution of vitD_3 in the LC-MS/MS analysis. The proton, sodium and ammonium adducts were selected for product ion scan that was run by using different combinations of eluent additives. Only the protonated adduct produced a product ion with significant abundance, and the other adducts remained intact during the collision, as is seen in Table 1.

Table 1. The abundance of the proton ($[M+H]^+$), sodium ($[M+Na]^+$) and ammonium ($[M+NH_4]^+$, $[M+H+NH_4]^{2+}$) adduct of vitamin D_3 at different combinations of ammonium formate and formic acid in the eluent. These were determined by using a product ion scan from *m/z* 150 - 600, collision energy 10. $HCOONH_4$ = ammonium formate, FA = formic acid.

When no additives were added to the eluent, the most abundant ion was the sodium adduct. Adding ammonium formate alone increased the abundance of the protonated adduct significantly and its product ion (*m/z* 298.1) was increased more than 40 times, whereas the ammonium adducts were reduced by a factor of ~10, while the sodium adduct was not affected. Adding formic acid alone decreased the abundance of all the ions by at least 50%. Adding both ammonium formate and formic acid to the eluent maintained the effect of formic acid on the sodium and ammonium adducts, while the effect of ammonium formate on the protonated adducts and its product ion was significantly reduced.

3.3. The effect of $^{13}C_5$-labelled vitamin D_3 as an internal standard

^{13}C-labelled internal standards have been shown to be superior to deuterium-labelled standards as they co-elute with their corresponding analytes, whereby ME is significantly decreased (Zhou et al., 2017). We showed that deuterium-labelled standards elute slightly before their corresponding unlabelled standards, which was not the case for ^{13}C-labelled standards (SM, Figure S5). Thus, the %RME was determined using vitD_3-[$^{13}C_5$] as an

internal standard. The %RME in each in-house reference material (n=3) of salmon, cheese, pork fat, egg yolk, and beef was 108 ± 6%, 100 ± 3%, 103 ± 4%, 102 ± 10%, and 99 ± 2% for vitamin D_3, respectively. For analysis of 25(OH)D_3 (n=3), it was 103 ± 1%, 103 ± 4%, 103 ± 3%, 104 ± 2%, and 102 ± 3% for the same food matrices, respectively. The method is also suitable for analysis of vitamin D_2 and 25(OH)D_2 and provides acceptable ME when using vitD_3-[$^{13}C_5$] and 25(OH)D_3-[$^{13}C_5$] as internal standards, respectively (SM, Table S6).

3.4. Comparison between the HPLC-UV-DAD and the LC-MS/MS methods

As part of the quality assurance of the analytical method in our laboratory, we include analyses of in-house reference samples, standardised reference materials, and participate in proficiency tests. The food matrices covered are fish, milk and dairy products, meat, and egg. The results from these analyses performed with the previously described methods were compared for vitD_3 (Table 2).

Table 2. Content of vitamin D_3 (µg/100 g) in different in-house reference samples as determined by the different methods.

Even though we do not have a complete dataset for all samples, we would like to focus on similarities as well as differences between the methods. In salmon, no difference was identified between the UV- and MS/MS-methods, but the precision for the MS-$^{13}C_5$-$HCOONH_4$ improved compared to the other methods. In cheese, similar levels were found for all four MS/MS-methods, as well as the trueness in the ISO17025 accredited methods documented for the standardised reference materials (NIST 1549a). Furthermore, infant formula has been the preferred matrix in proficiency testing in which our accredited methods, i.e., UV-DAD, MS-d_6-$HCOONH_4$, as well as MS-$^{13}C_5$-$HCOONH_4$, show a z-score between -1 and 1. For meat samples, no significant differences were identified between the MS/MS-methods (Pork fat 2014) or to the UV-method (Pork fat 2010), but in pork meat differences occurred. Our value (2.5 ng/g) for the content of vitD_3 in meat (NIST 1546a) obtained by the MS-d_6-CH_3NH_2/HCOOH method was similar to others (Roseland et al., 2016). Our results from MS-d_6-$HCOONH_4$ and MS-$^{13}C_5$-$HCOONH_4$ are lower than the reference value. We investigated the effect of prolonged LC run (18 min), and the MS-d_6-$HCOONH_4$ method analysed the content of vitD_3 being 1.9 ng/g, whereas the addition of 0.05% of formic acid as an additive provided results of 2.1 ng/g. When we repeated the same experiment using vitD_3-[$^{13}C_5$] as an internal standard, we did not see any significant

difference in the results between the ordinary method (10 min) and the two longer ones (18 min). These results indicate that MS-d_6-$HCOONH_4$ is not robust, and emphasize the importance that reduction of interferences may be achieved by prolonging the gradient and equilibration time of the runs. There might still be a challenge in the accuracy of the method in meat samples, as it is peculiar that using the same extraction procedure that was used in MS-d_6-CH_3NH_2/HCOOH seems to provide the correct results, but not when using vitD_3-[$^{13}C_5$] as internal standard and ammonium formate as additive.

In egg yolk, the UV-DAD and MS-d_6-CH_3NH_2/HCOOH gave comparable results. The change to the MS-d_6-$HCOONH_4$ method introduced the necessity to correct for recovery to avoid an overestimation of around 35%. As mentioned earlier, when using the ^{13}C-labelled internal standard for vitD_3, this bias was eliminated (%RME at 102%). From the results of vitD_3 in our in-house reference materials (Egg 2012 and Egg 2015), no differences are shown between the UV- and the MS-d_6-$HCOONH_4$-methods, but the content in the samples run with MS-d_6-$HCOONH_4$ (incl. formic acid) and MS-^{13}C-$HCOONH_4$ was 10% higher. The whole egg powder (NIST 1845a) was included in an inter-laboratory trial in which we participated using the MS-d_6-CH_3NH_2/HCOOH-method. In this study the level was assessed to 44.9 ± 4.5 ng/g, and thus our value was acceptable. However, the current standardised value is ~10% higher. This level indicated that our methods have bias, which our validation does not justify (Roseland et al., 2016; SRM 1845a; 2018).

The accuracy obtained with our old UV-DAD method relied on extensive clean-up and preparative HPLC until the full absorption spectra of vitamin D_3/vitamin D_2 were shown to be completely pure when measured using a DAD (Jakobsen et al., 2004). This procedure protected against interferences that would not be observed if the peaks were only recorded using one wavelength, as reported by Byrdwell (2009). These results indicate that UV-DAD is credible for quantification of vitamin D vitamers in food, even though LC-MS/MS methods are preferred due to smaller sample amount needed and the lower volumes of solvents used for extraction.

Comparing the content of 25(OH)D_3 analysed by the different methods (Table 3), there is the same tendency as for vitD_3, i.e., no difference for salmon, whole milk, and cheese, while differences might occur for fat, meat, and egg.

Table 3. Content of 25(OH)D_3 (µg/100 g) in different in-house reference samples as determined by the different methods.

The above comparison between methods based on UV-detection and MS/MS-detection shows that thorough validation that include the food products of interest is essential.

The minor difference in retention times between $vitD_3$ and $vitD_3$-[d_6] affected accuracy in quantitative analysis of $vitD_3$ due to the elution of these two compounds in the regions of different ion suppression. The use of $vitD_3$-[d_6] in continuous post-column infusion experiments gave insight into the effects that the use of various eluent additives has on the ion suppression. The food matrices studied originated from fish, dairy, meat and egg. We observed challenges in quantification of $vitD_3$ in these food matrices, with special attention on meat and eggs. The results from the post-column infusion gave insight into the effect of additives, pH, and of percentage of methanol. The information obtained enabled the optimisation of the method using deuterium-labelled internal standards, though it was not possible to omit correction for recovery for all matrices. The origin of the ion suppression is probably partly from additive in the plastic that are used in the clean-up steps, e.g single-use plastic tubes, plastic Pasteur pipettes, and pipette tips; however, these interferences elute after the end of the $vitD_3$ peak. Thus, omitting plastic utensils will not remove the ion suppression. Adding 0.05% formic acid to the eluent decreased the ion suppression, and thereby the difference in the ionization efficiency of the $vitD_3$ analogues was decreased, but with an effect on the abundance. This information, as well as the prolongation of the gradient, is useful if unforeseen results in quality control samples appear.

In the current standardised MS/MS-method for quantification of vitamin D validated for infant formula and adult nutritionals, deuterium-labelled vitamin D is used as an internal standard (ISO, 2018). Carbon-labelled internal standards have the same retention times as their corresponding unlabelled standards, and should be prioritized in the analyses of vitamin D in food. Such a method is more precise and is expected to obtain a true value.

Our use of in-house reference materials shows a consistency in the level of vitamin D using UV-DAD-detection and MS/MS-detection. The results of our study emphasize the need for a standardisation of vitamin D vitamers in foods to agree on trueness, especially in meat and egg matrices.

4. Conclusion

In this work, we successfully reported the use of a deuterated stable isotopically labelled internal standard in a post-column infusion experiment when a blank matrix is not available. By using post-column infusion with vitD_3-[d_6], we could evaluate the effect that different eluent additives have on ion suppression in extracts of different food matrices. Despite a thorough sample preparation consisting of saponification, LLE, SPE, and derivatisation with PTAD, we observed an enormous amount of ion suppression, which influence the abundance of vitD_3. Due to its shorter retention time, a deuterated vitD_3 internal standard cannot entirely eliminate ME when analysing vitD_3 in most food matrices. In contrast, carbon-labelled internal standards enabled simultaneous elution of vitD_3 and 25(OH)D_3 and their corresponding internal standard, resulting in recoveries at 100% and a robust chromatography. Our use of in-house reference materials show a consistency on the level of vitamin D using UV-DAD-detection and MS/MS-detection. The results emphasize the need for a standardisation of vitamin D vitamers analysis in food to agree on trueness, especially for meat and egg-products.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful to our colleague Henrik Lauritz Frandsen from DTU Food for providing equipment for post-column chromatographic runs and for the great discussion.

References

Barnkob, L. L., Petersen, P. M., Nielsen, J. P., & Jakobsen, J. (2019). Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities. *European Food Research and Technology*, *245*(2), 411–418. https://doi.org/10.1007/s00217-018-3173-6

Berg, T., & Strand, D. H. (2011). 13C labelled internal standards-A solution to minimize ion suppression effects in liquid chromatography-tandem mass spectrometry analyses of drugs in biological samples? *Journal of Chromatography A*, *1218*(52), 9366–9374. https://doi.org/10.1016/j.chroma.2011.10.081

Bilodeau, L., Dufresne, G., Deeks, J., Clément, G., Bertrand, J., Turcotte, S., Robichaud, A., Beraldin, F., Fouquet, A. (2011). Determination of vitamin D3 and 25-hydroxyvitamin D3 in foodstuffs by HPLC UV-DAD and LC-MS/MS. *Journal of Food Composition and Analysis*, *24*(3), 441–448. https://doi.org/10.1016/j.jfca.2010.08.002

Bonfiglio, R., King, R. C., Olah, T. V., & Merkle, K. (1999). The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Communications in Mass Spectrometry*, *13*(12), 1175–1185. https://doi.org/10.1002/(SICI)1097-0231(19990630)13:12<1175::AID-RCM639>3.0.CO;2-0

Buhrman, D. L., Price, P. I., & Rudewicz, P. J. (1996). Quantitation of SR 27417 in human plasma using electrospray liquid chromatography-tandem mass spectrometry: A study of ion suppression. *Journal of the American Society for Mass Spectrometry*, *7*(11), 1099–1105. https://doi.org/10.1016/S1044-0305(96)00072-4

Burild, A., Frandsen, H. L., Poulsen, M., & Jakobsen, J. (2014). Quantification of physiological levels of vitamin D3 and 25-hydroxyvitamin D3 in porcine fat and liver in subgram sample sizes. *Journal of Separation Science*, *37*(19), 2659–2663. https://doi.org/10.1002/jssc.201400548

Byrdwell, W. C. (2009). Comparison of analysis of vitamin D3 in foods using ultraviolet and mass spectrometric detection. *Journal of Agricultural and Food Chemistry*, *57*(6), 2135–2146. https://doi.org/10.1021/jf803398u

Byrdwell, W. C., Horst, R. L., Phillips, K. M., Holden, J. M., Patterson, K. Y., Harnly, J. M., & Exler, J. (2013). Vitamin D levels in fish and shellfish determined by liquid chromatography with ultraviolet detection and mass spectrometry. *Journal of Food Composition and Analysis*, *30*(2), 109–119. https://doi.org/10.1016/j.jfca.2013.01.005

CEN. (2009). *Foodstuffs - Determination of vitamin D by high performance liquid chromatography - Measurement of cholecalciferol (D3) or ergocalciferol (D2)*. 1–24. Retrieved from www.en-

standard.eu on the 25th of September 2020.

Dams, R., Benijts, T., Günther, W., Lambert, W., & De Leenheer, A. (2002). Influence of the eluent composition on the ionization efficiency for morphine of pneumatically assisted electrospray, atmospheric-pressure chemical ionization and sonic spray. *Rapid Communications in Mass Spectrometry*, *16*(11), 1072–1077. https://doi.org/10.1002/rcm.683

Dimartino, G. (2009). Simultaneous determination of cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) in foods by selected reaction monitoring. *Journal of AOAC International*, *92*(2), 511–517. https://doi.org/10.1093/jaoac/92.2.511

Egaas, E., & Lambertsen, G. (1979). Naturally occuring vitamin D3 in fish products analyzed by HPLC, using vitamin D2 as an international standard. *International Journal for Vitamin and Nutrition Research*, *49*, 35–42.

Furey, A., Moriarty, M., Bane, V., Kinsella, B., & Lehane, M. (2013). Ion supression; A critical review on causes , evaluation , prevention and applications. *Talanta*, *115*, 104–122. https://doi.org/10.1016/j.talanta.2013.03.048

Gomes, F. P., Shaw, P. N., Whitfield, K., & Hewavitharana, A. K. (2015). Simultaneous quantitative analysis of eight vitamin D analogues in milk using liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta*, *891*, 211–220. https://doi.org/10.1016/j.aca.2015.08.017

Heudi, O., Trisconi, M.-J., & Blake, C.-J. (2004). Simultaneous quantification of Vitamins A, D3 and E in fortified infant formulae by liquid chromatography-mass spectrometry. *Journal of Chromatography A*, *1022*(1–2), 115–123. https://doi.org/10.1016/j.chroma.2003.09.062

Höller, U., Quintana, A. P., Gössl, R., Olszewski, K., Riss, G., Schattner, A., & Nunes, C. S. (2010). Rapid determination of 25-hydroxy vitamin D3 in swine tissue using an isotope dilution HPLC-MS assay. *Journal of Chromatography B*, *878*(13–14), 963–968. https://doi.org/10.1016/j.jchromb.2010.02.026

ISO20636:2018. (2018). *Infant formula and adult nutritionals - Determination of vitamin D by liquid chromatography-mass spectrometry*. Retrieved from www.iso.org on the 25th of September 2020.

Jakobsen, J., Clausen, I., Leth, T., & Ovesen, L. (2004). A new method for the determination of vitamin D3 and 25-hydroxyvitamin D3 in meat. *Journal of Food Composition and Analysis*, *17*(6), 777–787. https://doi.org/10.1016/j.jfca.2003.10.012

Jakobsen, J., Maribo, H., Bysted, A., Sommer, H. M., & Hels, O. (2007). 25-Hydroxyvitamin D3 affects vitamin D status similar to vitamin D3 in pigs – but the meat produced has a lower content of vitamin D. *British Journal of Nutrition*, *98*(05), 908–913. https://doi.org/10.1017/S0007114507756933

Jakobsen, J., & Saxholt, E. (2009). Vitamin D metabolites in bovine milk and butter. *Journal of Food Composition and Analysis*, *22*(5), 472–478. https://doi.org/10.1016/j.jfca.2009.01.010

Konermann, L. (2017). Addressing a common misconception: Ammonium acetate as neutral pH "buffer" for native electrospray mass spectrometry. *Journal of the American Society for Mass Spectrometry*, *28*(9), 1827–1835. https://doi.org/10.1007/s13361-017-1739-3

Lipkie, T. E., Janasch, A., Cooper, B. R., Hohman, E. E., Weaver, C. M., & Ferruzzi, M. G. (2013). Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B*, *932*, 6–11. https://doi.org/10.1016/j.jchromb.2013.05.029

Mattila, P., Piironen, V., Uusi-Rauva, E., & Koivistoinen, P. (1995). Cholecalciferol and 25-hydroxycholecalciferol contents in fish and fish products. *Journal of Food Composition & Analysis*, Vol. 8, pp. 232–243. https://doi.org/10.1006/jfca.1995.1017

Matuszewski, B. K. (2006). Standard line slopes as a measure of a relative matrix effect in quantitative HPLC-MS bioanalysis. *Journal of Chromatography B*, *830*, 293–300. https://doi.org/10.1016/j.jchromb.2005.11.009

Matuszewski, B. K., Constanzer, M. L., & Chavez-Eng, C. M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC−MS/MS. *Analytical Chemistry*, *75*(13), 3019–3030. https://doi.org/10.1021/ac020361s

NMKL167. (2000). *Cholecalciferol (vitamin D3) and Ergocalciferol (vitamin D2). Determination by HPLC in foodstuffs*. Retrieved from www.nmkl.org on the 25th of September 2020.

Roseland, J. M., Patterson, K. Y., Andrews, K. W., Phillips, K. M., Phillips, M. M., Pehrsson, P. R., … Taylor, C. L. (2016). Interlaboratory Trial for Measurement of Vitamin D and 25-Hydroxyvitamin D [25(OH)D] in Foods and a Dietary Supplement Using Liquid Chromatography-Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, *64*(16), 3167–3175. https://doi.org/10.1021/acs.jafc.5b05016

SRM 1546a; Vitamin D_3 and 25-hydroxyvitamin D_3, Natl. Inst. Stand. Technol. U.S. Dep. Commer. Gaithersburg, MD. 21 March 2016.

SRM 1845a; Vitamin D_3 and 25-hydroxyvitamin D_3. *National Institute of Standards and Technology; U.S. Department of Commerce: Gaithersburg, MD*, 07 May 2018.

SRM 1549a; Vitamin D_3 and 25-hydroxyvitamin D_3, Natl. Inst. Stand. Technol. U.S. Dep. Commer. Gaithersburg, MD. 23 August 2018.

Strobel, N., Buddhadasa, S., Adorno, P., Stockham, K., & Greenfield, H. (2013). Vitamin D and 25-hydroxyvitamin D determination in meats by LC-IT-MS. *Food Chemistry*, *138*, 1042–1047. https://doi.org/10.1016/j.foodchem.2012.08.041

Trenerry, V. C., Plozza, T., Caridi, D., & Murphy, S. (2011). The determination of vitamin D3 in bovine milk by liquid chromatography mass spectrometry. *Food Chemistry*, *125*(4), 1314–1319. https://doi.org/10.1016/j.foodchem.2010.09.097

Trufelli, H., Palma, P., Famiglini, G., & Cappiello, A. (2011). An overview of matrix effects in liquid

chromatography-mass spectrometry. *Mass Spectrometry Reviews*, *30*(3), 491–509. https://doi.org/10.1002/mas.20298

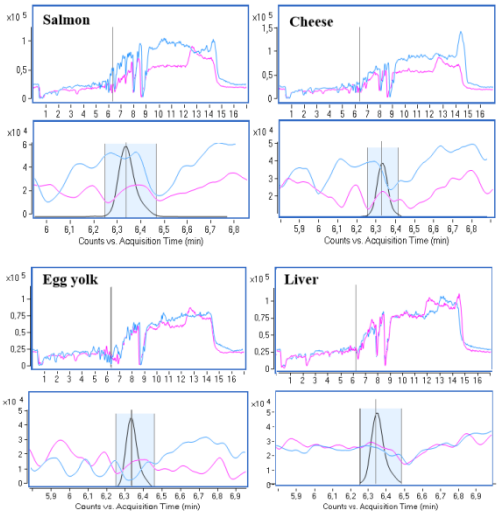
Van Berkel, G. J., Zhou, F., & Aronson, J. T. (1997). Changes in bulk solution pH caused by the inherent controlled-current electrolytic process of an electrospray ion source. *International Journal of Mass Spectrometry and Ion Processes*, *162*(1–3), 55–67. https://doi.org/10.1016/s0168-1176(96)04476-x

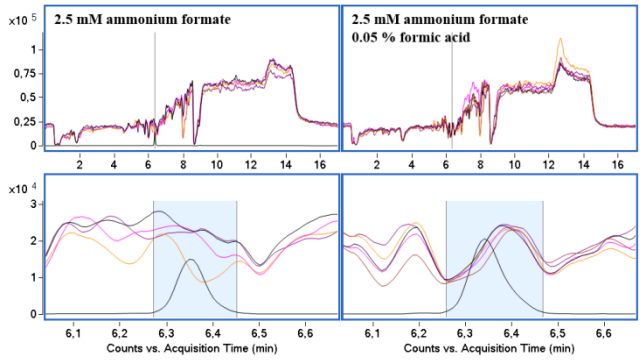
Wang, S., Cyronak, M., & Yang, E. (2007). Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, *43*(2), 701–707. https://doi.org/10.1016/j.jpba.2006.08.010

Wieling, J. (2006). LC-MS-MS experiences with internal standards. *Chromatographia*, *55*(S1), S107–S113. https://doi.org/10.1007/bf02493365

Wu, J., Wiegand, R., LoRusso, P., & Li, J. (2013). A stable isotope-labeled internal standard is essential for correcting for the interindividual variability in the recovery of lapatinib from cancer patient plasma in quantitative LC-MS/MS analysis. *Journal of Chromatography B*, *941*, 100–108. https://doi.org/10.1016/j.jchromb.2013.10.011

Zhou, W., Yang, S., & Wang, P. G. (2017). Matrix effects and application of matrix effect factor. *Bioanalysis*, *9*(23), 1839–1844. https://doi.org/10.4155/bio-2017-0214





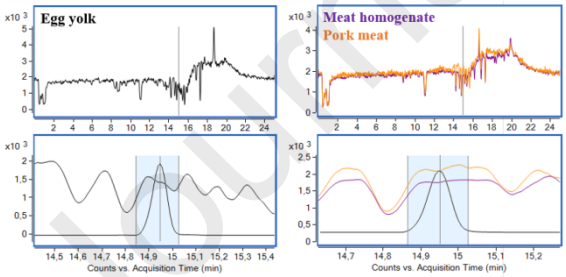


Table 1. The abundance of the proton ($[M+H]^+$), sodium ($[M+Na]^+$) and ammonium ($[M+NH_4]^+$, $[M+H+NH_4]^{2+}$) adduct of vitamin D_3 at different combinations of ammonium formate and formic acid in the eluent. Determined by using a product ion scan from *m/z* 150 - 600, collision energy 10. $HCOONH_4$ = ammonium formate, FA = formic acid.

			Abundance (x 10^4)				
Parent ion			$[M+H]^+$		$[M+Na]^+$	$[M+NH_4]^+$	$[M+H+NH_4]^{2+}$
Product ion			298.1	560.3	582.3	577.3	289.2
0 mM $HCOONH_4$	+	0% FA	2.0	2.4	14	2.3	3.3
2.5 mM $HCOONH_4$	+	0% FA	88	11	17	0.15	0.37
2.5 mM $HCOONH_4$	+	0.05% FA	9.1	2.8	5.7	1.6	1.1
0 mM $HCOONH_4$	+	0.05% FA	1.0	1.1	5.7	1.4	2.1

Table 2. Content of vitamin D_3 in different in-house reference samples as determined by the five different methods

Detection principle		**HPLC-DAD/UV**				**LC-MS/MS**					
Internal standard		**Vitamin D_2**		**vitD_3-[d_6]**		**vitD_3-[d_6]**		**vitD_3-[d_6]**		**vitD_3**	
Modifier 1		**none**		**Methylamine**		**NH_4-formate**		**NH_4-formate**		**NH_4-**	
Modifier 2		**none**		**formic acid**		**none**		**formic acid**		**none**	
Food matrix	**Sample type**	**vitD_3 (ng/g)**	**n**	**vitD_3 (ng/g)**	**n**	**vitD_3 (ng/g)**	**n**	**vitD_3 (ng/g)**	**n**	**vitD_3 (ng/g**	

Salmon	Inhouse-2014	154 ± 2.7[a]	5	155 ± 7.4[a]	7	143 ± 16[a]	4	154 ± 8.5[a]	4	150 ±
Cheese	Inhouse-2014	-		1.1 ± 0.1[a]	11	1.0 ± 0.1[a]	23	1.1 ± 0.1[a]	4	1.1 ±
Whole Milk	NIST 1549a	-		-		1.64	1	-		1.73 ±
Pork fat	Inhouse-2010	5.7 ± 0.4[a]	9	5.4 ± 0.8[a]	28	-		-		-
Pork fat	Inhouse-2014	-		3.6 ± 0.1[a]	4	4.1 ± 0.7[a]	27	4.1; 4.1	2	4.1 ±
Pork meat	Inhouse-2006	0.15±0.03[a]	13	-		0.11 ± 0.02[b]	7	-		-
Meat	NIST 1546a	-		2.5; 2.5	2	1.54 ± 0.1[a]	9	-		1.55 ±
Egg yolk	Inhouse-2012	26.4 ± 2.0[a]	16	27.2 ± 0.5[a]	3	26.4; 26,4[*#]	2	-		-
Egg yolk	Inhouse-2015	27.7±1.8[ab]	7	25.9; 26.3	2	27.2 ± 1.4[a#]	14	29.3 ± 1.9[bc]	6	30.0 ±
Whole Egg	NIST 1845a	-		39.8 ± 2.4[ab]	5	38.0 ± 0.5[b#]	4	-		40.5 ±

Different letters (a,b, c) in a row indicate significant differences ($p < 0.05$), t-test was performed when only two different methods were used for the same food sample. When n<3, the group is not included in statistical analysis.

n.r. – no reference value

[#]The results were corrected for factor 1.3 based on the recovery and matrix effect studies

[¤]Current reference value, however lower reference value was in use until 2018 (Roseland et al., 2016)

[*]SRM 1549a, 2018

[**]SRM 1546a, 2016

[***]SRM 1845a, 2018

Table 3. Content of $25(OH)D_3$ in different in-house reference samples as determined by the five different methods

Detection principle		**HPLC-DAD/UV**		**LC-MS/MS**						
Internal standard		**25(OH)D_2**		**25(OH)D_3-[d_6]**		**25(OH)D_3-[d_6]**		**25(OH)D_3-[d_6]**		**25(OH)**
Modifier 1		**none**		**Methylamine**		**NH_4-formate**		**NH_4-formate**		**NH_4-for**
Modifier 2		**none**		**formic acid**		**none**		**formic acid**		**none**
Food matrix	**Sample type**	**25(OH)D_3 (ng/g)**	**n**	**25(OH)D_3 (ng/g)**	**n**	**25(OH)D_3 (ng/g)**	**n**	**25(OH)D_3 (ng/g)**	**n**	**25(OH) (ng/g)**
Salmon	Inhouse-2014	3.4, 3.0	2	2.6 ± 0.1[a]	8	3.0 ± 0.3[a]	4	3.0; 3.1	2	2.8 ± 0.3
Cheese	Inhouse-2014	-		0.68 ± 0.04[a]	9	0.78 ± 0.1[a]	26	-		0.78 ± 0.
Whole Milk	NIST 1549a	-		-		0.72 ± 0.05[a]	9	-		0.72 ± 0.
Pork fat	Inhouse-2010	2.3 ± 0.1[a]	9	2.3 ± 0.1[a]	28	-		-		-
Pork fat	Inhouse-2014	-		2.1 ± 0.1[a]	4	2.2 ± 0.2[a]	38	2.56; 2.59	2	2.5 ± 0.3
Pork meat	Inhouse-2006	1.3 ± 0.09[a]	14	-		1.2 ± 0.2[b]	7	-		-
Meat	NIST 1546a	-		1.2 ± 0.1[a]	4	0.64 ± 0.09[b]	9	-		0.97 ± 0.
Egg yolk	Inhouse-2012	8.3 ± 0.9[a]	13	10.9 ± 0.2[b]	3	8.17, 7.99	2	-		-
Egg yolk	Inhouse-2015	9.4 ± 1.1[a]	3	-		9.4 ± 0.6[a]	24	9.0 ±0.3[a]	4	9.1 ± 0.2
Whole Egg	NIST 1845a	-		14.5 ± 2.5[a]	5	10.9 ±0.8[b]	11	-		11.5 ± 0.

Different letters (a,b, c) in a row indicate significant differences ($p < 0.05$), t-test was performed when only two different methods were used for the same food sample. When $n<3$, the group is not included in statistical analysis.

n.r. – no reference value

*SRM 1549a, 2018

**SRM 1546a, 2016

***SRM 1845a, 2018

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement:

Petra Ložnjak Švarc: conceptualization, methodology, validation, investigation, statistical analysis, visualization, writing – reviewing and editing. **Line Lundbæk Barnkob:** conceptualization, methodology, validation, investigation, writing – original draft, writing – reviewing and editing. **Jette Jakobsen:** conceptualization, methodology, validation, writing – reviewing and editing, supervision, project administration, funding acquisition.

Highlights

- Post-column infusion of vitD_3-[d_6] can be used to evaluate ion suppression
- Combination of food sample and eluent additives affects matrix effect
- Use of vitD_3-[d_6] does not guarantee correction of ESI-MS/MS matrix effect
- The additive ammonium formate increases the ionization of vitD_3
- ^{13}C-vitD should be part of a future standardised method for vitD in food