analytical chemistry

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Development and Validation of a Sensitive UPLC-ESI-MS/MS Method for the Simultaneous Quantification of 15 Endocannabinoids and Related Compounds in Milk and Other Biofluids

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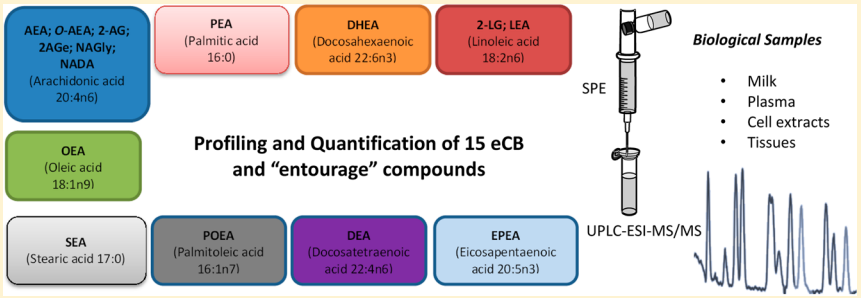
Supporting Information



ABSTRACT: The endocannabinoid (eCB) system has gained an increasing interest over the past decades since the discovery of anandamide and 2-arachidonoyl glycerol (2-AG). These, and structurally related compounds, are associated with a wide variety of physiological processes. For instance, eCB levels in milk have been associated with infants' feeding and sleeping behavior. A method based on ultraperformance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC−ESI-MS/MS) was developed and validated for the simultaneous quantification of 15 eCBs and related compounds, including both fatty acid amides and glycerols. Linearity ($0.9845 < R^2 < 1$), limit of detection and quantification (0.52−293 pg on column), inter- and intraday accuracy (>70%) and precision (CV < 15%), stability, and recovery (in milk and plasma) were established in accordance to the U.S. Food and Drug Administration guidelines. The method was successfully applied to bovine and elk milk revealing species-specific eCB profiles, with significant different levels of 2-AG, 2-linoleoyl glycerol, docosahexaenoyl ethanolamide, palmitoyl ethanolamide, and oleoyl ethanolamide. Furthermore, stearoyl ethanolamide and docosatetraenoyl ethanolamide were only detected in elk milk. In summary, our UPLC−ESI-MS/MS method may be used for quantification of eCBs and related compounds in different biofluids and applied to investigations of the role of these emerging compounds in various physiological processes.

Endocannabinoids (eCBs) belong to a family of endogenous lipid-related signaling molecules able to bind to and activate cannabinoid receptors (CB1 and CB2), including 2-arachidonoyl glycerol (2-AG), *N*-arachidonoyl ethanolamine (anandamide, AEA), *O*-arachidonoyl ethanolamine (virodhamine, *O*-AEA), *N*-arachidonoyl dopamine (NADA), and 2-arachidonyl glycerol ether (noladin ether, 2-AGe).[1] The best-studied and most active eCBs are 2-AG and AEA, discovered in the 1990s.[2,3] AEA is a conjugate of arachidonic acid with ethanolamine, categorized as a fatty acid amide, whereas 2-AG is an arachidonic acid glycerol ester (Figure 1).

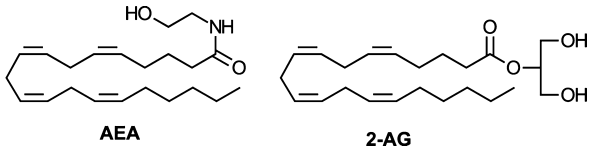


Figure 1. Chemical structures of *N*-arachidonoyl ethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG).

AEA and 2-AG are synthesized on demand in various tissues in multiple biochemical pathways, catalyzed by *N*-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) for the production of AEA and the *sn*-1-specific diacylglycerol lipase α and β (DAGL α and β) for the production of 2-AG.[4] eCBs are released from cell membrane phospholipid precursors rather than being released from intracellular stores. After their release and binding to CB receptors, eCBs are removed by membrane transport and degraded. The degradation pathways are catalyzed by monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) for 2-AG and AEA, respectively.[5] These highly orchestrated eCB synthesis and degradation processes create a delicate extra- and intracellular balance, reflected in physiological concentrations.

Received: October 10, 2013
Accepted: December 10, 2013
Published: December 10, 2013

Other fatty acid amides and glycerol esters have been characterized as "cannabimimetic" when capable of activating CB1 or CB2 (totally or partially)[1,6] or entourage compounds because they do not activate CB receptors but can modify the activity of eCBs by, for instance, inhibiting their degradation via competition for catabolic enzymes such as FAAH and MAGL or by having affinity for other receptors such as transient receptor potential vanilloid type-1 (TRPV1),[7] orphan G-coupled receptors (GPR55, GPR18, and GPR119), or peroxisome proliferator-activated receptor α (PPARα).[5,8]

Fatty acid amides such as *N*-acylethanolamines (NAEs) are associated with the regulation of inflammation and pain.[9,10] NAEs include AEA, linolenoyl ethanolamide (LEA), oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA), docosatetraenoyl ethanolamide (DEA), stearoyl ethanolamide (SEA), eicosapentaenoyl ethanolamide (EPEA), docosahexaenoyl ethanolamide (DHEA), and palmitoleoyl ethanolamide (POEA). 2-AG and 2-linoleoylglycerol (2-LG) are fatty acid glycerol derivatives, while arachidonoylglycine (NAGly) is an AEA derivate. All of the above, together with *O*-AEA, NADA and 2-AGe, were analyzed in a single chromatographic run. The method was validated and successfully applied to milk samples for investigation of species-specific levels of eCBs and related compounds.

Levels of eCBs in milk are associated with infants' feeding and sleeping behavior. In particular, 2-AG stimulates the newborn to initiate milk intake and is present in higher concentrations than AEA in human milk just after birth.[11] The presence of other fatty acid glycerols, besides 2-AG, such as 2-LG and 2-palmitoyl glycerol (2-PG), which are not active on the CB receptors, were found to have an "entourage effect", enhancing 2-AG activity and milk intake.[12] NAEs, such as OEA, AEA, and PEA, have also been detected in human and bovine milk, but in lower levels than 2-AG.[13]

Furthermore, the eCB system plays a role in a variety of other physiological processes with the modulation of eCB levels associated with diseases such as cancer,[14] cardiovascular disorders,[15] mood disorders,[16] memory loss, schizophrenia,[17] and also with nutritional factors.[5] Besides milk, eCBs have been detected in a wide variety of biological matrixes, such as human plasma,[18,19] serum,[20] mammalian and human brain tissues,[21] cell cultures, and reproductive system fluids.[4]

Different extraction protocols have been reported for isolating eCBs and related compounds from biological samples including liquid−liquid extraction (LLE) using chloroform/methanol mixtures and/or toluene, as well as solid-phase extraction (SPE).[22] The use of SPE has resulted in superior sensitivity compared to LLE, with lower limits of quantification (LOQ) and smaller sample volumes required. eCBs have limited stability in common working conditions (for example, they react with plastic), in particular, 2-AG, which spontaneously isomerizes via acyl migration to 1-AG in a pH-dependent manner.[1] Thus, analytical procedures, from the point of sample collection and onward, must be carefully optimized regarding parameters such as storage conditions, extraction solvent, pH, and concentration procedures. The presence of 1-AG in biological samples remains difficult to attribute to endogenous or postsampling formation from 2-AG isomerization.

Several methods have been reported for quantification of eCBs in biological samples, but it is a challenging task mainly because they are found in picomolar to nanomolar levels, which puts high demands on the analytical method and equipment of choice.[1] Furthermore, the levels found in different studies can be quite different for the same eCB analyte in the same biological matrix, and it is not clear if this large concentration range is due to biological or instrumental variability, which reinforces the need for a robust and sensitive analytical method and equipment.[1]

The most common analytical methods for eCB quantification are high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) or fluorescence detection, as well as ultraperformance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS), and gas chromatography (GC) coupled with MS/MS.[1] The lack of strong chromophores or fluorescent groups in eCBs, as well as the potential of MS to use stable-isotope-labeled analogues of the analytes to be quantified together with the accuracy, precision, and sensitivity of chromatographic techniques and MS operated in the multiple reaction monitoring mode (MRM), have made GC/MS/MS and UPLC−MS/MS methods central in eCB quantifications. For instance, Fride et al.[12] and Di Marzo et al.[23] used GC/MS/MS to quantify eCBs and related compounds in bovine, goat, and human milk, and 2-AG, AEA, 2-PG, 2-LG, and OEA were detected and quantified.

Early methods described for quantifying eCBs were performed with GC/MS/MS, which required sample prederivatization (silylation or acylation) resulting in time-consuming analyses. Furthermore, this procedure may lead to discrepancies in results, for instance, in the recovery rates. Recently, eCBs have been analyzed without the need of chemical derivatization by using LC instead of GC coupled to triple-quadrupole MS/MS with atmospheric pressure ionization techniques, such as electrospray ionization (ESI)[19] and atmospheric pressure chemical ionization (APCI).[24] Increased sensitivity of AEA detection by LC−ESI-MS/MS analysis was described by the addition of silver acetate to the mobile phase, producing AEA adducts $[AEA + Ag]^+$.[25] However, this method meant that the ion source had to be cleaned at multiple occasions due to contamination with silver ions, which is a disadvantage when running large batches of samples in high-throughput studies.

Chromatographic separation of eCBs is commonly achieved using reversed-phase C_{18}/C_8 columns, either with isocratic mixtures of methanol/water with high percentage of organic solvent or in a gradient mode with increasing amounts of organic solvent (acetonitrile or methanol).[1] Ammonium acetate or formic acid are often used as buffers.

The aim of this study was to develop and validate a sensitive, precise, selective, and accurate UPLC−ESI-MS/MS method to quantify 15 eCBs and related compounds in different biological samples. The compounds selected for inclusion in our method were derivatives of nine fatty acids, arachidonic acid (20:4n6), oleic acid (18:1n9), linoleic acid (18:2n6), docosahexaenoic acid (22:6n3), palmitic acid (16:0), stearic acid (17:0), eicosapentaenoic acid (20:5n3), docosatetraenoic acid (22:4n6), and palmitoleic acid (16:1n7), based on their potential involvement in the eCB system, and thereby of importance in a large number of physiological processes and diseases. The validated method was successfully applied to milk for investigation of species-specific levels of eCBs and related compounds.

MATERIAL AND METHODS

Chemicals and Reagents. AEA, AEA-d_8, 2-AG, 2-AG-d_8, *O*-AEA, 2-AGe, NADA, PEA, OEA, OEA-d_4, DEA, NAGly, EPEA, DHEA, DHEA-d_4, POEA, LEA, SEA, and 2-LG (Figure

dx.doi.org/10.1021/ac403352e | *Anal. Chem.* 2014, 86, 1186−1195

S1, Supporting Information) were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Acetonitrile (ACN) and methanol (MeOH) were from Merck (Darmstadt, Germany). Ammonium acetate (CH_3COONH_4) was purchased from Scharlau Chemie (Barcelona, Spain). All solvents and chemicals were of HPLC grade or higher. Water was purified by a Milli-Q Gradient system (Millipore, Milford, MA, U.S.A.).

Internal Standards (IS). Deuterated compounds (AEA-d_8, 2-AG-d_8, and OEA-d_4, IS) were used as internal quantification standards and were added to samples before extraction to mimic the extraction of the endogenous compounds. For each native compound, a suitable IS was selected based on structural similarities; AEA-d_8 was used as IS for AEA and *O*-AEA, 2-AG-d_8 was used for 2-AG, 2-AGe, and 2-LG, whereas OEA-d_4 was used for the remaining compounds (Table S1, Supporting Information). DHEA-d_4 was used to calculate recovery rates of IS and was added in the last step before analysis.[26]

Standard Stock Solutions. Analytical standards were used as ready-made standard stock solutions or as solutions prepared from solid substances and stored at −80 °C. 2-AG, *O*-AEA, 2-AGe, and 2-LG were prepared and stored in ACN, and the other standards were prepared and stored in ethanol to yield a final stock solution concentration of 250 μg/mL. Stock solutions of IS were prepared to reach a final concentration of 40 μg/mL.

Standard Curve Preparation. Further dilutions of each stock solution were made with methanol at 10 different calibration levels, prepared fresh on a weekly basis and stored at −80 °C (Tables S2 and S3, Supporting Information). The lowest concentration in the calibration curve was further diluted for limit of quantification (LOQ) and detection (LOD) purposes.

UPLC−ESI-MS/MS Instrumentation. The UPLC system consisted of a Waters Acquity Ultra Performance equipment (Milford, MA, U.S.A.) with a binary pump, a thermostatted column compartment, and an autosampler. Injection volume was 10 μL, and LC separation was achieved using a Waters BEH C_{18} column (2.1 mm × 150 mm, 2.5 μm particle size). Additional columns used for optimization purposes can be found in Table S4 (Supporting Information). The column was maintained at 60 °C. A gradient mobile phase consisting of Milli-Q water (A) and MeOH with 10 mM CH_3COONH_4 (B) was used under the following conditions: 0.0−9.0 min 79% B, 9.0−9.5 min 79−90% B, 9.5−10.5 min 90% B, 10.5−14.0 min 79% B, at a flow rate of 0.4 mL/min. The autosampler temperature was maintained at 10 °C.

The mass analysis was done on a Waters triple-quadrupole MS (Micromass Quattro Ultima) equipped with an electrospray ionization source operating in positive mode (ESI^+). N_2 was used as drying gas (60 L/h) and Ar as nebulization gas (650 L/h). Source and desolvation temperatures were 150 and 350 °C, respectively. For each standard the precursor ion $[M + H]^+$ was determined in MS^1 full scan experiments, and the product ions in MS/MS experiments, subsequently monitored for each transition in MRM mode. Source parameters, such as cone voltage (CV), collision energy (CE), and capillary voltage (CAP) were optimized for each MRM transition with the actual column eluent composition and flow. Data were acquired by MassLynx workstation acquisition software (version 4.1, Waters).

The method was validated according to the U.S. Food and Drug Administration (FDA) guidelines over three consecutive days for linearity, LOD, LOQ, inter- and intraday accuracy and precision, recovery, and stability.[27]

Linearity. Triplicates of each standard were prepared and analyzed at 10 different concentrations. Calibration curves were calculated by the least-squares linear regression method with equal weighting factor using the equation $y = m(x) + b$, where "y" is equal to the response ratios (native standard peak area/internal standard peak area), "m" is equal to the slope of the calibration curve, "x" is equal to the on column concentration of the native analyte, and "b" is equal to the y-interception of the calibration curve. For concentration determinations of each analyte in real samples, the peak area ratio between the analyte and its corresponding internal standard (Table S1, Supporting Information) was used, and the concentrations were calculated using the calibration curve equation.

Limit of Detection and Limit of Quantification. LOD and LOQ of each standard were defined as the concentration at which the signal-to-noise ratio (S/N) was equal to or greater than 3 and 10, respectively.

Accuracy and Precision. Accuracy and precision were determined using quality control samples (QC) of 100 mM phosphate buffer saline (PBS) spiked with four different concentration levels (8.11, 64.9, 325, and 8110 pg/μL on column) of each native standard. Three replicates of each QC sample were extracted by SPE as described below (in the Extraction of Endocannabinoids section) and analyzed by LC−MS/MS in the same batch together with a complete set of calibration standards on three different days. The concentration of each QC sample was determined using the calibration curve obtained in each batch.

The intraday accuracy was determined as the percent difference between the mean concentration for each analytical run (n = 3) and the expected concentration. The interday accuracy was determined as the percent difference between the mean concentration on the three different days and the expected concentration (n = 9). The inter- and intraday accuracy was considered acceptable in the range of 80−120%.

The intra- and interday precision was obtained by calculating the coefficient of variation (% CV) for the mean concentration (n = 3 and n = 9, respectively).[26] The inter- and intraday precision was considered acceptable when the CV was less than 20%.

Recovery. An amount of 250 μL of 100 mM PBS was spiked with native and internal standard solutions at three different levels (0.584, 5.84, and 29.2 ng/μL) prior to extraction. The same procedure was repeated for internal standards in other biological matrixes (bovine and elk milk, human and fish plasma). Recovery rates for each internal standard were calculated using calibration curves obtained at five different concentrations normalized against DHEA-d_4 and expressed as the percentage of the expected value. The recovery was considered acceptable above 80%.

Stability. The stability of each analyte in bovine milk was tested by measuring the concentrations directly after sample collection and at different time intervals, after storage at different temperatures (−20 and −80 °C) and with two freeze−thaw cycles.

Working solution stability was determined by comparison of solutions freshly prepared and solutions stored at −80 °C for 4 weeks. Analytes were considered stable when 80−120% of the initial concentration was found.

Biological Application. Bovine animals (*Bos tarus*) of different breeds (Swedish Mountain, Swedish Friesian, and a

dx.doi.org/10.1021/ac403352e | *Anal. Chem.* 2014, 86, 1186−1195

Table 1. Mass Spectrometry Parameters for MRM Transitions [Retention Time (rt), Capillary Voltage (CAP), Cone Voltage (CV), and Collision Energy (CE)] and Linearity, Limit of Quantification (LOQ), and Limit of Detection (LOD) for the Analyzed Compounds (n = 3)

compound	abbreviation	rt/min	percursor ion	transition[a]	CAP/V	CV/V	CE/eV	R^2	slope (±SD)	LOD (pg on column)	LOQ (pg on column)	linear range pg/μL
arachidonoyl glycine	NAGLy	3.05	362	362 > 287	500	40	12	0.9997	0.053 ± 0.001	3.7	7.3	0.73−14610
				362 > 203	500	40	17					
eicosapentaenoyl ethanolamide	EPEA	3.70	346	346 > 62	500	37	14	0.9999	0.10 ± 0.006	1.8	3.6	0.36−14610
				346 > 201	500	37	12					
palmitoleoyl ethanolamide	POEA	4.08	298	298 > 62	500	47	14	0.9998	0.30 ± 0.01	6.1	12	0.036−146.1
				298 > 281	500	50	13					
docosahexaenoyl ethanolamide	DHEA	4.91	372	372 > 62	500	35	16	0.9999	0.022 ± 0.0007	9.1	18	1.83−14610
				372 > 311	250	50	10					
docosahexaenoyl ethanolamide-d_4	DHEA-d_4	4.88	376	376 > 66	500	50	20					
arachidonoyl ethanolamide	AEA	5.01	348	348 > 62	500	42	14	0.9999	0.41 ± 0.006	3.6	7.3	0.36−14610
				348 > 203	500	45	12					
arachidonoyl ethanolamide-d_8	AEA-d_8	5.00	356	356 > 63	250	40	12					
linolenoyl ethanolamide	LEA	5.01	324	324 > 62	500	50	16	0.9977	0.94 ± 0.01	0.52	1.2	0.12−14610
				324 > 245	500	55	12					
2-arachidonoyl glycerol	2-AG	5.47	379	379 > 269	500	45	16	0.9994	0.92 ± 0.03	7.3	18	1.8−14610
				379 > 287	500	50	14					
2-arachidonoyl glycerol-d_8	2-AG-d_8	5.44	387	387 > 294	250	60	18					
2-linoleoyl glycerol	2-LG	5.66	355	355 > 263	500	40	10	1	1.0 ± 0.05	146	293	29−14610
O-arachidonoyl ethanolamine	*O*-AEA	5.75	348	348 > 62	500	35	14	0.9996	0.25 ± 0.01	73	146	15−14610
				348 > 215	500	45	14					
N-arachidonyl dopamine	NADA	5.55	440	440 > 137	500	50	28	0.9845	0.51 ± 0.03	73	146	15−14610
				440 > 154	500	60	16					
palmitoyl ethanolamide	PEA	6.06	300	300 > 62	500	45	14	0.9999	0.084 ± 0.001	1.4	2.8	0.28−14610
				300 > 283	500	50	14					
docosatetraenoyl ethanolamide	DEA	6.21	376	376 > 62	500	40	16	0.9999	0.10 ± 0.002	0.52	0.73	0.073−14610
				376 > 315	500	55	12					
oleoyl ethanolamide	OEA	7.24	326	326 > 62	500	50	14	0.9997	0.26 ± 0.008	0.52	1.2	0.12−14610
				326 > 309	500	50	14					
oleoyl ethanolamide-d_4	OEA-d_4	7.24	330	330 > 66	250	40	18					
2-arachidonoyl glycerol ether	2-AGe	7.42	382[b]	382 > 273	500	30	10	0.9999	2.9 ± 0.2	7.3	18	1.8−14610
				382 > 121	500	30	16					
stearoyl ethanolamide	SEA	12.75	328	328 > 62	500	40	24	0.9998	0.46 ± 0.001	1.4	2.8	0.28−14610
				328 > 311								

[a]The first transition on each row was used for quantification and the second for qualification purposes. [b]$[M + NH_4]^+$.

dx.doi.org/10.1021/ac403352e | Anal. Chem. 2014, 86, 1186−1195

combination of Swedish Friesian with Red and White cattle) were chosen from a farm in Northern Sweden, approved for delivery of milk to the local dairy industry. Eurasian elks (*Alces alces*) from a farm in the same region were also chosen for milk collection.

Sample Collection. The bovine milk was collected from 10 animals in polypropylene tubes during early morning, and kept on dry ice until analysis on the same day. The same procedure was done for milk collection from two elks on two different days (morning and evening), except analysis was done 20 days later and samples were kept at −80 °C.

Extraction of Endocannabinoids. A previously published SPE protocol was adapted for sample preparation.[4] Briefly, extraction of analytes from bovine and elk milk was achieved by SPE using Waters Oasis HLB cartridges (60 mg of sorbent, 30 μm particle size), washed with 5 mL of ethyl acetate, followed by 10 mL of MeOH, and then conditioned with 10 mL of wash solution (30% MeOH). Bovine milk (30 mL) and elk milk (10 mL) were centrifuged at 3000*g* for 10 min at room temperature, and the lower phase (~30 and 9 g, bovine and elk milk, respectively) was loaded onto the SPE cartridges and spiked with 10 μL of internal standard solution (3 μg/mL). The sample was allowed to pass through the cartridges, 5 mL of wash solution was applied, and the cartridges were dried under high vacuum before the analytes were eluted into polypropylene tubes with 5 mL of ACN, followed by 2 mL of MeOH and 1 mL of ethyl acetate. To completely remove the remaining solvents from the cartridges, high vacuum was applied for several minutes. Each polypropylene tube contained 6 μL of 30% glycerol in MeOH to serve as a trap solution for the analytes. The solvents were evaporated with a MiniVac system (Farmingdale, NY, U.S.A.), and the analytes were reconstituted in 100 μL of methanol and vortexed, and if necessary centrifuged to remove any residuals. Solutions were then transferred to LC vials with low-volume inserts, 10 μL of recovery standard (DHEA-d_4) was added, and UPLC−ESI-MS/MS analysis was performed immediately.

The upper phase obtained from the milk centrifugation was extracted by different methods such as LLE (methanol/chloroform and toluene) and SPE. However, it was not possible to detect any of the analytes in this fraction.

Statistical Analysis. Species-specific analyte levels were calculated and expressed as mean ± SEM using the GraphPad Prism 6 (San Diego, CA, U.S.A.). Differences between groups for each compound were analyzed using the post hoc Tukey's multiple comparison test with $p < 0.05$ considered significant.

RESULTS AND DISCUSSION

Method Development. A robust and sensitive UPLC−ESI-MS/MS method was developed and validated for profiling of eCBs and related compounds, biosynthesized mainly from arachidonic acid and other polyunsaturated fatty acids, and present in diverse biological samples. A few issues regarding the analysis of these compounds include chemical instability, complexity of sample matrixes, structural similarities, and the low biological concentration levels, which necessitate a highly sensitive analytical method. Part of the analytical challenge is to find an optimal compromise between the number of analytes, sensitivity, specificity, and accuracy to be able to accomplish the goals regarding the biological samples to be analyzed.

Separation Optimization. The UPLC method was optimized to ensure resolution among all standards, including critical pairs of isomers such as AEA/*O*-AEA and 2-AG/1-AG (Figure S2, Supporting Information). These compounds share the same molecular weight and have identical MRM transitions in the ESI^+ experiments and are therefore difficult to separate. AEA and *O*-AEA chromatographic separation is required since they are both endogenous compounds, but unlike AEA, *O*-AEA is a full agonist of the CB2 receptor and a partial agonist of the CB1 receptor. Therefore, their individual quantification is an advantage.[28] Furthermore, 1-AG activity on CB receptors is not conclusive, but since 2-AG undergoes rapid isomerization by acyl migration to 1-AG under common experimental conditions, adequate chromatographic separation is needed to determine the true biological 2-AG concentrations.[1] For most of the samples analyzed, the amount of 1-AG detected was below the LOD and never above LOQ. Among the tested columns (Table S4, Supporting Information), the Waters BEH C_{18} column provided the best resolution between critical pairs of isomers (AEA/*O*-AEA and 2-AG/1-AG) and was therefore selected for all subsequent analyses.

Different organic solvents are reported for the composition of mobile phases used for LC−MS analysis of eCBs.[1] The most commonly used are MeOH and ACN combined with Milli-Q water. These solvents were tested, and it was found that MeOH provided a superior ionization and less ion suppression over ACN for the majority of the compounds. The addition of organic modifiers (formic acid, acetic acid, ammonium acetate, among others) has been reported to increase the ionization of eCB.[1] Formic acid and ammonium acetate were tested in the mobile phase, resulting in higher ion intensity with ammonium acetate.

The optimal UPLC conditions, column temperature, gradient composition, and pH were selected based on the resolution and intensity of each peak. The retention time precision for each compound was 98−101% ($n = 10$).

MS/MS Optimization. The protonated molecular ions for all compounds were determined in full-scan MS experiments over a range of $50 < m/z < 500$ by triple-quadrupole ESI^+. The protonated molecular ion $[M + H]^+$ and the most intense adduct ion, for each standard, are shown in Table S5 (Supporting Information). The most intense fragment ion in the product ion spectrum for each standard was selected to be monitored in MRM mode. The Na^+ adducts are very resistant to fragmentation and therefore not so useful for MS/MS analysis.

The selection of the most intense fragment ion for each standard was complex since the fatty acid moieties allow for fragmentation into many products. The fragment ion at m/z 62 was the most intense fragment for ethanolamide derivatives due to the fragmentation of the amide bond (Figure S1, Supporting Information).

CAP, CE, and CV were optimized for MRM transitions of each $[M + H]^+$ ion (Table 1). Representative examples of the extracted MRM chromatograms obtained from a standard mixture and from bovine and elk milk are show in Figure 2. No cross-talk between channels that were used for monitoring standards, internal standards, and recovery standard were observed.

Validation. The main goal of the method development was to provide a robust and sensitive analytical protocol to quantify a large array of eCBs and related compounds in different biological samples. The use of the biological sample as matrix (i.e., plasma or milk) to prepare calibration curves was not viable since eCBs are endogenous compounds and their baseline levels would interfere with the calibration concen-

dx.doi.org/10.1021/ac403352e | *Anal. Chem.* 2014, 86, 1186−1195

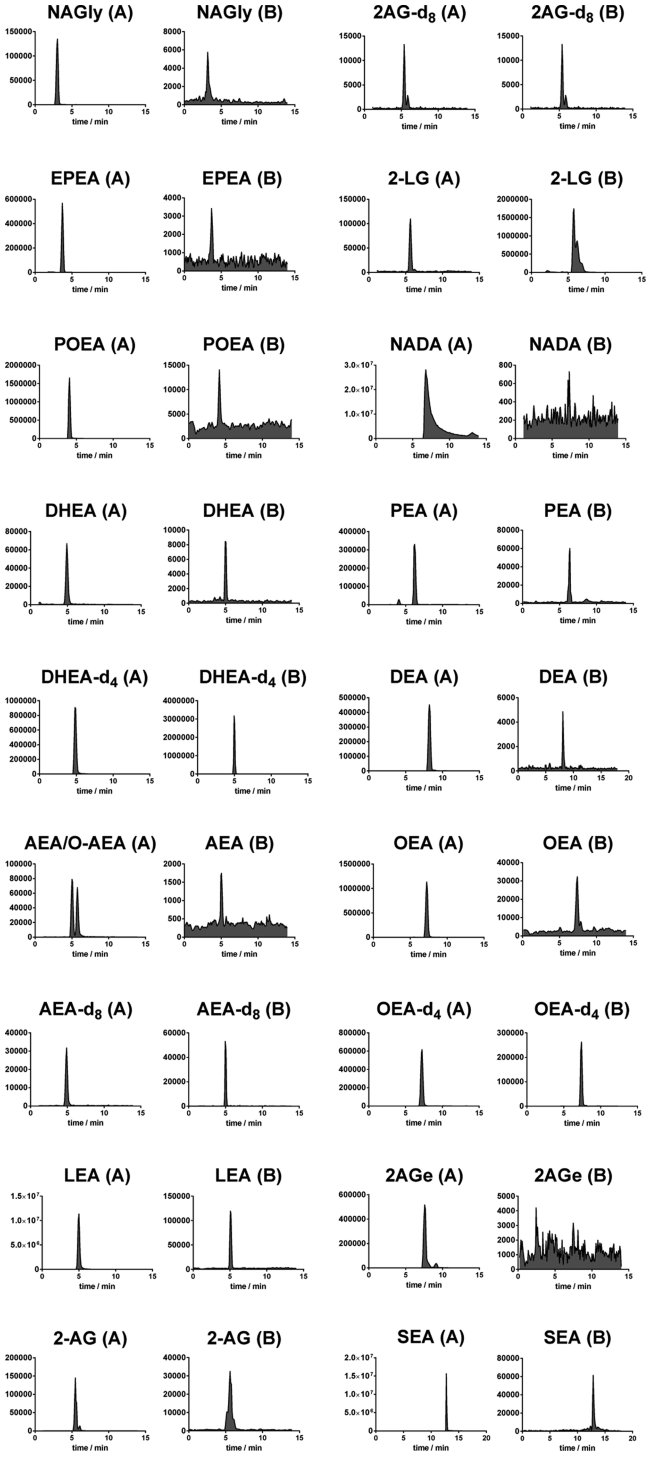


Figure 2. MRM chromatograms of each analyte analyzed in a standard solution mixture (A) and in a bovine milk sample (B), except DEA and SEA extracted from an elk milk sample.

trations. Therefore, we used a 100 mM PBS solution for validation purposes with calibration curves prepared in methanol.

Linearity, LOD, and LOQ. The linearity of the method was determined with calibration curves over a concentration range of 0.0365−14610 pg/μL at 10 different levels for each standard. Regression analysis produced R^2 values above 0.999, with only two exceptions (Table 1). No weighting factors were used.

LOD and LOQ of each compound were defined as the concentration at which a signal-to-noise ratio of at least 3 (LOD) and 10 (LOQ) was achieved. The LOD ranged between 0.52 and 146 pg on column, whereas LOQ ranged between 0.73 and 293 pg on column (Table 1).

Several studies have previously reported quantifications of a subset of eCBs, such as 2-AG and common NAEs (AEA, PEA, SEA, and DEA).[19,29−31] However, to our knowledge, this is the first method to simultaneously quantify as many as 15 eCBs, including both NAEs and fatty acid glycerols (AEA, 2-AG, *O*-AEA, 2-AGe, NADA, PEA, OEA, DEA, NAGly, EPEA, DHEA, POEA, LEA, SEA, and 2-LG) in a single analytical run using UPLC−ESI-MS/MS. Williams et al.[24] reported a less sensitive HPLC−APCI-MS/MS method analyzing 15 standards, including both eCBs and free fatty acids. However, *O*-AEA, NADA, and 2-LG were not included in that method. Furthermore, Balvers et al.[18] also reported a HPLC−ESI-MS/MS method with 12 eCB standards included, but it was also less sensitive and chromatographic separation between 2-AG and 1-AG was not achieved. Only *O*-AEA was detected with a lower LOQ compared to our method.

Accuracy and Precision. Triplicate injections of each QC sample were analyzed to establish intraday variation (Table 2). Furthermore, QC samples were run on three different days to determine interday variation. For each day, calibration curves were prepared and run together with the QC samples. As mentioned above, accurate calibration curves prepared in plasma are difficult to obtain due to the presence of endogenous compounds. To overcome this issue we prepared calibration curves in methanol according to previously reported protocols.[24]

Accuracy and precision parameters in line within the FDA guidelines (accuracy in the range of 100 ± 20% and CV values below 20%, representing precision) across the range of concentrations tested (at four different levels) for intra- and interday analysis of QC samples are presented in Table 2. The majority (70%) of the QC samples showed an accuracy within 80−110% and a precision of 0.5% < CV < 15%. The lowest level of concentration, QC4, displayed a wider range of accuracy (75−154%). For 2-LG, *O*-AEA, NADA, DHEA, and 2-AGe it was not possible to establish the accuracy and precision for the QC with the lowest concentration because it was below LOQ (ND in Table 2).

Recovery. Recovery is commonly assessed by measuring analyte levels in native samples, and in samples spiked with the analytes of interest (standard addition method), and by calculating the difference in analyte levels in spiked and native sample. Any deviating results from the expected outcome (based on the spiking concentration) indicate recovery rates above or below 100% (e.g., due to analyte losses and volume inaccuracy). However, when spiking low amounts of eCBs to samples with endogenous eCB content, overestimation of the recovery rate may occur.[32] A more convenient and precise method is to determine the recovery of deuterated IS spiked to each sample, assuming similar physiochemical properties of deuterated and native compounds. Recovery of IS ranged from 74% to 114% in different matrixes (PBS, human plasma, fish plasma, bovine milk, and elk milk), with CV ≤ 20% (Figure 3). The recovery rates for native eCBs in PBS compared in general well with those found for the IS (Table S6, Supporting Information).

Stability. The stability tests in bovine milk analyzed at different time points after collection showed that the analytes

dx.doi.org/10.1021/ac403352e | *Anal. Chem.* 2014, 86, 1186−1195

Table 2. Intra- and Interday Precision and Accuracy for Quality Control (QC) Samples at Four Different Concentration Levels[a]

	QC1 8.10 ng/μL			QC2 325 pg/μL			QC3 64.9 pg/μL			QC4 8.11 pg/μL		
	mean ng/μL	accuracy %	precision %	mean pg/μL	accuracy %	precision %	mean pg/μL	accuracy %	precision %	mean pg/μL	accuracy %	precision %
NAGly												
intraday 1	7.38	91	1.2	251	77	9.4	54.1	84	4.2	6.10	75	6.6
intraday 2	6.55	80	3.2	239	74	2.1	60.4	93	2.9	8.12	100	12
intraday 3	6.41	79	3.5	265	82	2.2	52.1	80	7.0	7.39	92	15
interday	6.80	84	16	230	71	13	56.0	86	8.8	7.23	89	14
EPEA												
intraday 1	6.73	82	1.9	303	93	4.6	77.0	118	2.5	9.90	123	9.0
intraday 2	6.94	85	1.7	350	108	3.5	73.4	112	6.7	9.78	120	11
intraday 3	5.90	73	4.5	375	115	12	75.9	117	3.7	9.69	119	9.2
interday	6.61	81	7.4	343	106	10	75.1	116	7.9	9.83	121	12
POEA												
intraday 1	7.17	89	0.87	326	100	4.7	69.3	107	1.0	8.17	101	6.5
intraday 2	7.62	94	3.3	334	103	4.1	77.9	120	0.6	9.41	116	4.1
intraday 3	5.90	73	3.3	320	98	4.1	70.1	108	0.6	9.09	112	4.1
interday	7.17	89	5.6	319	98	7.2	68.7	106	16	8.81	108	11
DHEA												
intraday 1	9.32	115	4.5	293	90	2.9	76.1	117	1.2	ND	ND	ND
intraday 2	10.0	124	4.4	323	99	2.7	68.9	106	5.2	ND	ND	ND
intraday 3	8.01	98	11	318	98	13	66.3	102	5.8	ND	ND	ND
interday	9.13	113	14	313	96	6.8	75.4	116	12	ND	ND	ND
AEA												
intraday 1	8.45	104	0.9	327	109	6.5	75.2	128	11	9.41	117	6.8
intraday 2	8.15	99	5.0	325	106	5.5	82.4	130	6.2	12.5	154	11
intraday 3	7.69	93	12	329	130	10	79.0	113	15	9.10	113	7.8
interday	8.23	101	8.5	327	116	12	78.9	127	19	10.2	127	8.8
LEA												
intraday 1	6.40	79	1.6	285	88	0.9	70.3	108	5.0	8.79	107	2.7
intraday 2	6.41	79	4.9	283	87	2.3	67.8	104	4.3	9.20	113	4.3
intraday 3	6.90	85	7.7	262	81	6.1	69.1	107	4.4	9.61	118	3.6
interday	6.29	78	6.8	277	85	5.5	69.0	106	4.3	9.38	115	4.9
2-AG												
intraday 1	8.66	108	7.7	355	110	0.8	72.1	110	4.8	8.30	102	7.9
intraday 2	8.62	106	0.5	357	110	2.3	70.2	108	7.1	8.59	106	8.6
intraday 3	8.37	103	5.4	345	106	10	69.3	107	6.1	8.85	108	7.2
interday	8.71	108	6.8	353	109	5.5	70.7	109	5.5	8.6	106	9.5
2-LG												
intraday 1	5.81	72	7.9	256	79	2.9	ND	ND	ND	ND	ND	ND
intraday 2	6.40	79	3.1	249	77	5.0	ND	ND	ND	ND	ND	ND
intraday 3	6.13	75	4.2	289	89	12	ND	ND	ND	ND	ND	ND
interday	5.85	72	5.3	285	88	11	ND	ND	ND	ND	ND	ND
O-AEA												
intraday 1	8.28	102	0.66	268	82	6.3	ND	ND	ND	ND	ND	ND
intraday 2	6.44	79	13	216	66	1.7	ND	ND	ND	ND	ND	ND
intraday 3	7.19	85	8.4	267	82	10	ND	ND	ND	ND	ND	ND
interday	7.50	92	12	259	80	6.5	ND	ND	ND	ND	ND	ND
NADA												
intraday 1	8.01	99	5.2	331	102	4.9	ND	ND	ND	ND	ND	ND
intraday 2	8.26	102	4.0	335	103	9.4	ND	ND	ND	ND	ND	ND
intraday 3	8.91	110	9.2	359	111	8.7	ND	ND	ND	ND	ND	ND
interday	8.63	105	8.7	340	105	6.9	ND	ND	ND	ND	ND	ND
PEA												
intraday 1	8.14	99	1.4	354	109	2.4	69.0	107	1.3	8.69	107	1.5
intraday 2	8.12	99	7.0	350	108	3.1	68.4	105	2.4	8.81	108	5.9
intraday 3	7.01	86	15	363	112	8.5	71.3	109	3.9	8.30	102	6.7
interday	8.07	99	4.1	357	110	4.8	70.8	110	3.4	8.71	107	10
DEA												
intraday 1	7.45	92	2.3	327	101	2.5	67.1	104	3.9	9.38	116	9.8
intraday 2	6.63	82	11	334	103	3.2	68.3	104	3.4	8.90	109	1.8
intraday 3	6.51	81	12	314	97	11	78.9	119	3.7	8.24	102	6.9

dx.doi.org/10.1021/ac403352e | *Anal. Chem.* 2014, 86, 1186−1195

Table 2. continued

	QC1 8.10 ng/μL			QC2 325 pg/μL			QC3 64.9 pg/μL			QC4 8.11 pg/μL		
	mean ng/μL	accuracy %	precision %	mean pg/μL	accuracy %	precision %	mean pg/μL	accuracy %	precision %	mean pg/μL	accuracy %	precision %
DEA												
interday	6.87	85	9.5	328	101	6.6	67.0	103	8.6	8.36	104	9.5
OEA												
intraday 1	7.30	89	1.6	313	96	5.8	69.2	107	7.0	8.22	101	5.5
intraday 2	6.67	83	8.8	333	102	2.5	69.1	106	4.5	8.68	107	2.3
intraday 3	6.91	86	14	302	93	5.9	68.6	104	2.4	9.90	122	3.7
interday	7.01	86	7.3	323	99	4.4	68.4	106	5.0	8.81	108	3.8
2-AGe												
intraday 1	6.93	86	8.6	336	104	0.5	76.0	117	7.8	ND	ND	ND
intraday 2	7.11	89	4.9	329	101	9.2	79.1	122	4.6	ND	ND	ND
intraday 3	6.91	85	7.1	358	110	15	71.5	110	3.9	ND	ND	ND
interday	6.72	82	9.5	337	104	9.0	75.4	116	12	ND	ND	ND
SEA												
intraday 1	8.21	102	1.4	322	99	9.3	70.0	107	5.8	7.67	95	3.5
intraday 2	8.32	102	4.2	332	102	6.8	67.9	105	6.1	7.51	92	4.8
intraday 3	7.71	96	2.5	344	106	7.2	71.3	109	4.6	8.34	102	11
interday	8.05	99	2.9	338	104	7.5	70.2	108	5.9	7.93	97	6.4

[a]ND: not determined (values below LOQ).

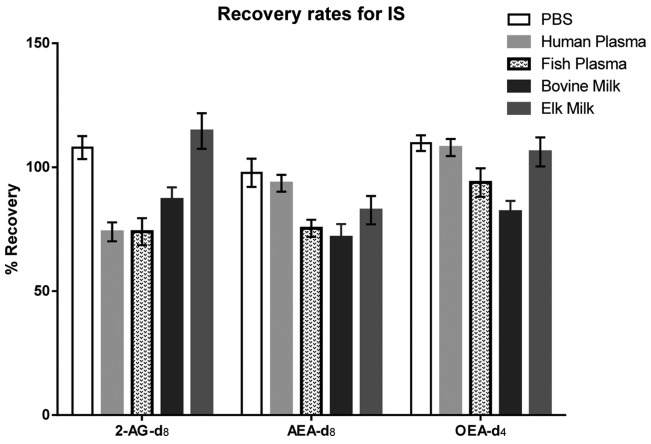


Figure 3. Recovery rates expressed as mean ± SEM for deuterated internal standards (IS) in PBS (n = 6), human plasma (n = 24), fish plasma (n = 6), bovine milk (n = 10), and elk milk (n = 3).

were stable, with levels within 80−120% of the initial levels, at −80 °C for at least 1 month (Table S7, Supporting Information). Two freeze−thaw cycles (samples frozen at −20 °C, thawed at room temperature, and refrozen again at −20 °C) were done during a time period of 1 week. Levels after two freeze−thaw cycles were much lower than those measured immediately after sample collection (Table S7, Supporting Information). This fact reinforces the need to establish well-defined protocols for eCB analysis, including the sample collection.

Working solutions were stable for 4 weeks at −80 °C, except for 2-AG, AEA, NAGly, and NADA. They displayed concentrations lower than 70% of the initial concentration when analysis was repeated after 4 weeks but were stable for 1 week at −80 °C. Therefore, standard solutions were prepared fresh on a weekly basis.

Biological Application. In order to investigate species-specific eCB profiles in milk, we applied the validated method to elk and bovine milk, obtained from three different breeds. 2-AG, 2-LG, AEA, PEA, OEA, NAGly, EPEA, DHEA, POEA, and LEA were detected in all samples, whereas DEA and SEA only were detected in elk milk (Figure 4). To our knowledge, none of them has been quantified in elk milk before, and only 2-AG, 2-LG, AEA, PEA, and OEA have previously been detected in bovine milk.[11−13,23] Hence, NAGLy, EPEA, DHEA, POEA, and LEA were for the first time quantified in bovine milk.

All concentrations can be found in the Supporting Information, Table S8 (corresponding to Figure 4). For the different bovine breeds, EPEA was found in the lowest concentration (24.6 ± 5.4 pg/g of milk) and 2-LG was found in the highest concentration (296 ± 39.6 pg/g milk). The levels found for 2-AG and 2-LG are in good agreement with those found with GC/MS in bovine milk. However, OEA was found in higher amounts than AEA and in considerably lower levels than 2-AG, in contrast to previous findings.[12] There were no significant differences between the cow breeds regarding the levels of different analytes, with exception of 2-LG and LEA (Figure 4).

For elk milk, PEA was found in the highest levels (1812 ± 290 pg/g milk) and DEA was found in the lowest levels (17.1 ± 10.9 pg/g milk). The levels of 2-AG, 2-LG, and DHEA were significantly lower in elk milk compared to bovine milk (all breeds), while the levels of PEA and OEA were significantly higher. These findings indicate that eCBs and related compounds in milk are produced in a species-specific manner, possibly due to different feeding behavior, leading to different profiles of fatty acid precursors and different eCB intake of the offspring. More research is needed to investigate if this is the case.

■ CONCLUSIONS

A UPLC−ESI-MS/MS method for the simultaneous quantification of 15 eCBs and related compounds was developed and validated for the purpose of investigating the role of these compounds in physiological processes. Validation parameters were established in accordance to FDA guidelines for linearity, LOD, LOQ, inter- and intraday accuracy and precision, recovery, and stability. Overall, the validation criteria were met and the method was considered sensitive and robust over

dx.doi.org/10.1021/ac403352e | Anal. Chem. 2014, 86, 1186−1195

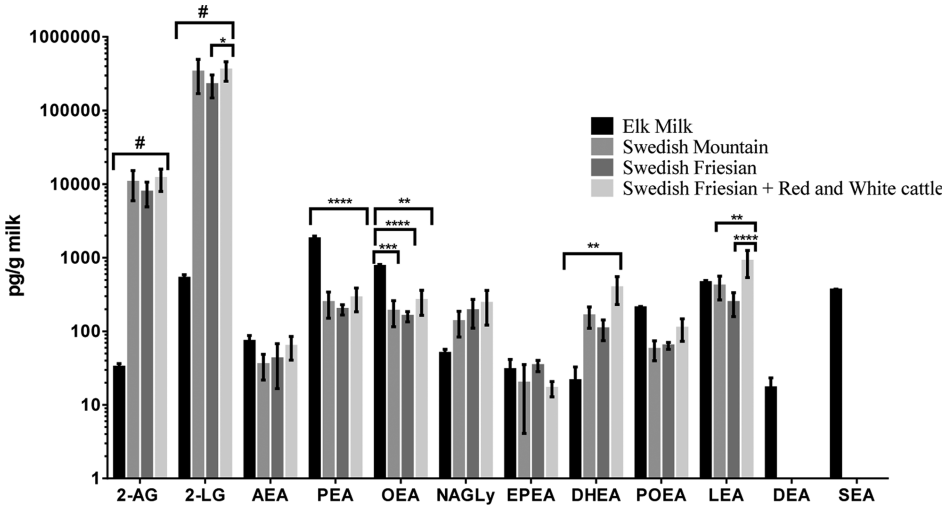


Figure 4. Levels of eCBs and related compounds in milk obtained from elk and three different cow breeds (Swedish Mountain, Swedish Friesian, and a combination of Swedish Friesian with Red and White cattle). All values represent the mean ± SEM (n = 3, except for Swedish Friesian with n = 4). # indicates a $p < 0.0001$ between elks and cow breeds, ****$p < 0.0001$, ***$p = 0.0002$, **$p = 0.005$, and *$p < 0.021$. The levels of individual metabolites are presented on a logarithmic scale since they varied over 4 orders of magnitude.

the calibration ranges established for each compound. The method was successfully applied in an investigation of species-specific milk concentrations showing significant different levels of 2-AG, 2-LG, DHEA, PEA, and OEA between elks and cows. Furthermore, SEA and DEA were only detected in elk milk. Only two analytes (2-LG and LEA) were significantly different between bovine breeds. The results imply that there are larger inter- than intraspecies variations in eCB levels. The method can easily be modified for application to other biofluids as demonstrated by similar recovery rates in milk and plasma (both human and fish). Further method development for analysis of eCBs and related compounds in plasma and other biological matrixes is currently under way.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The Swedish Research Councils Formas and Vinnova and the Kempe Foundations are gratefully acknowledged for financial support. Älgens Hus in Bjurholm is recognized for excellent assistance in sample collection.

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