JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Determination of Dapsone in Muscle Tissue and Milk Using High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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ABSTRACT: A precise and selective liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the determination of dapsone in muscle tissue and milk has been developed. The sample preparation was based on extraction with organic solvent and automated solid-phase extraction (SPE) cleanup. At least three product ions were monitored for the analyte. The method was validated according to the European Decision 2002/657/EC. Estimated analytical limits were 0.0018 ng/g for CCα and 0.0031 ng/g for CCβ in meat and milk. An excellent linear concentration range was observed for both matrices with a correlation coefficient better than 0.997. Recoveries were 105–117% in meat and 101–108% in milk, with satisfactory precision and coefficients of variance (CV) less than 8%. Additionally, a simplified quantification approach was successfully evaluated depending only on the response factor (F) without the use of calibration curve. The developed method provides reliable and sensitive identification and quantification of dapsone in meat and milk.

KEYWORDS: *dapsone, meat, milk, validation, LC-MS/MS, APCI*

INTRODUCTION

Dapsone (4-[(4-aminobenzene)sulfonyl]aniline) is a bacteriostatic and bactericidal sulfone derivative. The mechanism of the bacteriostatic action of dapsone is probably similar to that of the sulphonamides as both actions are inhibited by *para*-aminobenzoic acid.[1] In veterinary medicine, it has been used as an antibiotic for the treatment of infectious diseases in animals such as mastitis, coccidiosis, keratitis, otitis, and toxoplasmosis.[2] The use of dapsone can induce methemoglobinaemia in humans and animals by oxidizing the iron in hemoglobin from its ferrous to its ferric form, making the hemoglobin unable to carry oxygen to tissues. Furthermore, hemolysis and changes in oxygen affinity may occur, increasing the toxic symptoms more than would be expected from the methemoglobin concentrations alone.[3] Deaths associated with the administration of dapsone have been reported from agranulocytosis, aplastic anemia, and other blood dyscrasias.[4,5]

Therefore, to protect public health, the use of dapsone is forbidden in foodstuffs of animal origin according to the council regulation 37/2010.[6] A concentration of 5 ng/g has been recommended as the minimum required performance limit (MRPL) according to the EURL (European Union Reference Laboratory Berlin-Germany and Fougères-France, responsible for group substances A6) guidance paper.[7] The MRPL value is necessary for analytical purposes and method validation, although the substance is prohibited, and the CCα and the CCβ limits should always be below the MRPL set by the guidance paper. For nonauthorized-banned substances, a result can be considered noncompliant if it is higher than the CCα for confirmatory methods and the CCβ for screening methods. It is therefore necessary to have sensitive and specific analytical methods for the detection and confirmation of dapsone in animal products. The methods must be in compliance with the criteria of the Commission Decision 2002/657/EC, which set the requirements for the validation of methods.[8]

Several methods have been reported for the determination of dapsone in animal products. Hadjigeorgiou et al. developed a method for the determination of dapsone in meat and milk with liquid chromatography coupled to mass spectrometry (LC-MS/MS).[9] The sample preparation of the method included extraction with organic solvent and solid-phase extraction (SPE) achieving CCα and CCβ values at 0.12 and 0.16 μg/kg, respectively. Two methods have been developed for the determination of sulfonamides and dapsone in milk, one with LC-MS/MS[10] and one with high-performance liquid chromatography coupled to ultraviolet detector (HPLC-UV).[11] The LC-MS/MS method involved extraction with organic solvent and dilution and was capable of detecting dapsone at 1 μg/kg. Hela et al. used HPLC coupled to diode array detector (HPLC-DAD) for the detection of sulfonamides and dapsone in animal tissues.[12] Moreover, two multiresidue methods also containing dapsone were developed in meat by using LC-MS/MS[13] and high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry (LC-Q-TOF-MS).[14] The LC-MS/MS method included extraction with organic solvent and dilution with a CCa value of 3.3 μg/kg. The Q-TOF-MS method set a validation level of 10 μg/kg for dapsone by purifying the samples with extraction with organic solvent, dilution, and SPE. All LC-MS techniques applied electrospray ionization (ESI) for the formation of positive ions. Our group applied atmospheric pressure chemical ionization (APCI) as the ionization technique, providing higher detection sensitivity for our compound.

Received: September 12, 2011
Revised: December 2, 2011
Accepted: December 5, 2011
Published: December 5, 2011

The aim of the present study was to develop a sensitive method by applying a different ionization technique (APCI), enabling high specificity, quantification, and confirmation of the presence of dapsone in meat and milk at very low concentrations (trace levels). To achieve these ends, LC-MS/MS was selected as the analytical platform in combination with a simple and fast purification procedure using automated SPE.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Dapsone was purchased from Sigma (Sigma-Aldrich, Steinhem, Germany), and the internal standard dapsone-d8 was from TRC (Toronto Research Chemicals, Toronto, Canada). The structures of the compounds are shown in Figure 1.

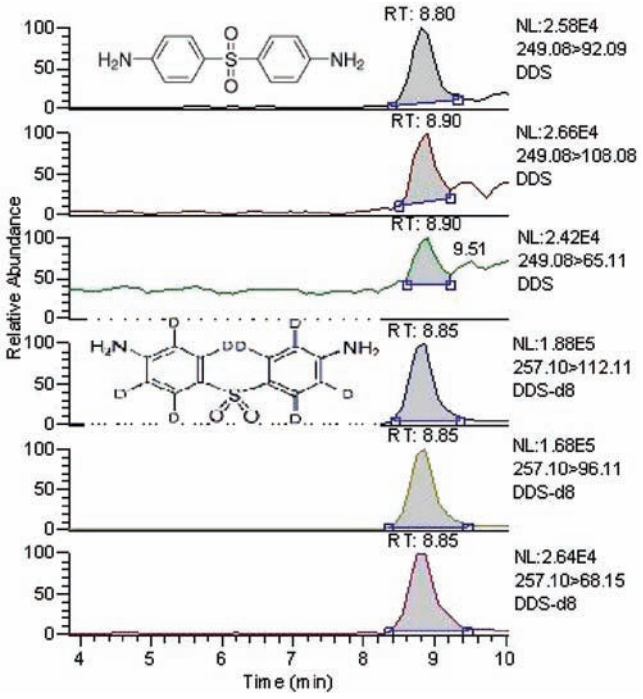

Figure 1. MRM chromatogram of a meat sample spiked with dapsone (three product ions are shown in the three upper panes) at a concentration of 0.01 ng/g, with dapsone-d8 (three product ions are shown in the three panes below) at a concentration of 0.3 ng/g.

Individual stock solutions were prepared in methanol ranging between 0.10 and 1 mg/mL and were stored at −20 °C. Working standard solutions were prepared in methanol by diluting the stock standard solutions into the needed range and stored at 4 °C.

Methanol and water were of LC-MS grade obtained from Merck (Darmstadt, Germany). All other organic solvents, acetonitrile, acetone, and *tert*-butyl methyl ether (TBME), were of LC-grade and purchased from Sigma (Sigma-Aldrich). Tris(hydroxymethyl) aminomethane and acetic acid were of analytical reagent grade and were obtained from Merck. Tris buffer, 0.1 M (pH 9.5), was prepared by dissolving 12.1 g of Tris in 1000 mL of LC-MS grade water.

Sampling. A variety of bovine muscle and raw milk samples were obtained from the local market and local farms. Samples of ca. 250 g for muscle tissue and 50 mL for milk were collected, and after the meat samples were minced, they were stored at −20 °C. For validation experiments, portions of these samples were individually analyzed to verify the absence of dapsone. Moreover, to ensure the robustness of the method, 20 different bovine meat and milk samples were also analyzed with the developed methodology.

Equipment. The LC-MS/MS system was a TSQ Quantum Ultra Accurate Mass coupled to a Finnigan Surveyor LC system (Thermo-Electron, San Jose, CA) operated under Xcalibur 2.1 software. A Zymark TurboVap LV Evaporator (Sparta, NJ) was applied for the evaporation of solvents and an ASPEC XL system (Gilson, United States) was used for automated SPE. ASPEC is designed to receive either up to 108 samples each with 100 mg of packing (1 mL) or up to 60 samples each with 500 or 200 mg of packing (6 or 3 mL), giving the opportunity of analyzing a large amount of samples daily. Method parameters are loaded into the software, and the system operates automatically. Oasis HLB (60 mg, 3 mL) SPE cartridges were obtained from Waters (Milford, MA).

APCI interface was selected for the ionization of the analyte with the discharge current set at 6 μA. The vaporizer temperature was set at 450 °C, and the capillary temperature was set at 290 °C. The sheath and auxiliary gas were 30 and 5 arbitrary units, respectively. The tube lens voltage was adjusted to 94 V for dapsone and 85 V for dapsone-d8. The collision energy was optimized for each compound, and the scan time was set at 500 ms for each product ion. Three product ions were selected for each precursor ion as given in Table 1.

Chromatography was performed on a Hypersil ODS analytical column, 150 mm × 4.6 mm, 4 μm (ThermoElectron). The column was maintained at 30 °C, and the tray of the autosampler was maintained at 18 °C. A gradient containing 1% acetic acid in water (A) and 100% methanol (B) was applied. Used was the following gradient: 0−6 min, 90% A (v/v); 6−10 min, 90−10% A; and 10−12 min, 10% A. From 12 to 16 min, the system was equilibrated with initial conditions (90% A, v/v). The flow rate was 0.7 mL/min, and the injection volume was 15 μL. The retention times for the compounds are shown in Table 1.

Extraction Procedure. Seven milliliters of 0.1 M Tris buffer was added to 5 g of minced meat, and the mixture was vortex mixed thoroughly for 30 s. Next, 10 mL of TBME was added, inverted several times for 10 min, and centrifuged at 3000 rpm for 5 min. The supernatant was collected and evaporated at 55 °C under a stream of nitrogen to dryness. The dry residue was dissolved with 4 mL of a mixture of water−methanol (9:1, v/v) and vortexed for 30 s.

Ten milliliters of acetonitrile was added to a 5 mL of milk sample, and the mixture was vortex mixed for 30 s, inverted several times for 10 min, and centrifuged at 3000 rpm for 5 min. The upper acetonitrile layer was collected and evaporated at 60 °C under a stream of nitrogen at a final volume of about 1 mL. Three milliliters of water−methanol (9:1, v/v) was added, and the mixture was vortexed for 30 s.

Table 1. Retention Time, Precursor and Product Ions of the Compounds, and CCα and CCβ Values

			m/z				
compd	R_t (min)	APCI	precursor	product	collision energy (eV)	CCα	CCβ
dapsone	8.80	positive	249.08	108.08[a]	21	0.0018	0.0031
				92.09[b]	20		
				65.11	41		
dapsone-d8	8.85	positive	257.10	112.11[ab]	19		
				96.11	21		
				68.15	29		

[a]Most intense ion. [b]Ion used for quantification.

dx.doi.org/10.1021/jf203701f | J. Agric.Food Chem. 2012, 60, 29−35

Table 2. Confirmation Based on the Ion Ratio and RRT for Six Spiked Meat Samples at a Concentration of 0.04 ng/g

analyte, dapsone; matrix, meat	reference		sample					
sample identification	standard		**1**	**2**	**3**	**4**	**5**	**6**
analyte signal 1 (base peak) 249.08 > 108.08	129		425	328	345	339	465	385
analyte signal 2 249.08 > 92.09	115		413	310	337	338	397	401
ion ratio (signal 2/signal 1)	0.891		0.972	0.945	0.977	0.997	0.854	1.042
tolerance ion ratio 1 ±(%) 20	***0.713***	***1.070***	IN	IN	IN	IN	IN	IN
identification points			4.0	4.0	4.0	4.0	4.0	4.0
conclusion			presence confirmed	presence confirmed	presence confirmed	presence confirmed	presence confirmed	presence confirmed
estimated concentration unit (ng/g)		0.04	~0.04	~0.04	~0.04	~0.04	~0.04	~0.04
comments								
RT internal standard (min)	8.95		8.55	8.55	8.55	8.55	8.55	8.55
RT analyte (min)	9.01		8.60	8.60	8.60	8.60	8.60	8.60
RRT analyte	1.007		1.006	1.006	1.006	1.006	1.006	1.006
tolerance RRT ± (%) 2.5	***0.982***	***1.032***	IN	IN	IN	IN	IN	IN

The above extracts from meat and milk samples were purified with the same procedure on Oasis HLB cartridges. The cartridge was conditioned with 3 mL of methanol and 3 mL of water. After sample loading, the cartridge was washed with 3 mL of water−methanol (95:05, v/v) and additionally with 3 mL of water−methanol (6:4, v/v). Then, the compounds were eluted from the cartridge with 3 mL of acetone. The solvent was dried under a stream of nitrogen at 55 °C. The analytes were reconstituted in 80 μL of methanol, and the resulting solution was transferred into an injection vial for further LC-MS/MS analysis. The automated SPE procedure was carried out on an ASPEC XL autosampler.

Statistical Analyses. The validation software ResVal version 2.1 was used for the calculation of the validation parameters. Data were evaluated with analysis of variance (ANOVA) at 95% confidence level by using the statistical software MINITAB. For the estimation of the uncertainty, a coverage factor of 2.33 was applied to achieve a confidence level of 99%.

The response factor (F), in chromatography and spectroscopy, is the ratio between a signal produced by an analyte and the quantity of analyte that produces the signal. The response factor (F) is the proportionality constant for the analyte. What this means is that regardless of the amounts of the analyte and the internal standard in solution, the ratio of the ratios of area to concentration will always yield a constant. In practice, a solution containing known amounts of both analyte and internal standard is injected into the LC system, and the F value is calculated. Then, a separate solution with an unknown amount of analyte and a known amount of internal standard is injected. The response factor is applied to the data from the second solution, and the unknown concentration of the analyte is found. The response factor, F, can be calculated with the following equation:

$$\frac{\text{area of analyte signal}}{\text{concentration of analyte}} = F\left(\frac{\text{area of standard signal}}{\text{concentration of standard}}\right)$$

$$\frac{A_x}{[X]} = F\left(\frac{A_s}{[S]}\right)$$

This method works well provided that the chemical properties of the standard are closely related to the chemical properties of the internal standard. As a result of this fact, the best choice is to use the deuterated analogue for the target analyte.[15]

RESULTS AND DISCUSSION

Extraction and Cleanup Optimization. Dapsone is a weak base (pK of 1.0) and practically insoluble in water. Our intention was to use an alkaline buffer to enable the extraction of the analyte from the meat matrix into the organic solvent. We tested two types of buffers, phosphate and Tris, in combination with TBME, methanol, ethylacetate, and acetonitrile. The same organic solvents were also applied directly to milk samples to cause precipitation of the proteins and simultaneously dissolve the analyte of interest. In terms of clean, separate phases and higher recoveries, the best result was achieved with the combination of Tris buffer and TBME for meat samples; for milk, the best result was obtained when using acetonitrile. Most methods reported in the literature apply acetonitrile for the extraction of dapsone from milk and meat samples[10,12−14] or carbonate buffer and dichloromethane as extraction solvent.[9]

SPE was applied to increase efficiency of the clean up procedure. Three SPE cartridges were evaluated (Strata-C18, Discovey-C18, and Oasis HLB) aiming to improve analyte recovery and removal of matrix interferences. Two washing steps were selected with a mixture of water and methanol to remove polar and less polar interferences. Washing with a solution of water−methanol (5:5, v/v) resulted in 20% loss in recovery for all SPE cartridges. For the elution of the analyte acetone, methanol and acetonitrile were tested. Acetonitrile resulted in less symmetric peak shapes. Elution with methanol and acetone resulted in comparable qualitative and quantitative results, and acetone was selected as it is faster to evaporate.

When applying the cleanup procedure to the three tested SPE cartridges, the Oasis HLB resulted in slightly higher recoveries (4−6% higher than the other cartridges) and provided more reproducible quantitative results. In previous studies, Silica gel columns and Oasis MCX and StrataX SPE cartridges have been used for sample purification prior to the analysis of dapsone.[9,10,14] To automate the SPE process and reach a high capacity methodology, an ASPEC system was applied. These systems offer total automation and control of the sample preparation process, including cleanup and concentration. Automation in sample preparation is regarded favorable for a number of reasons, which include reduction in economic and time cost, improvement in repeatability, and minimization of error sources.[16]

Method Validation. The applicability of the method for the analysis of dapsone in meat and milk was tested according to the 2002/657/EC requirements.[8] The specificity, matrix effect, linearity, accuracy, precision, analytical limits (CCα and CCβ), and stability of the method were estimated based on the analysis of spiked meat and milk samples with the analytes of

dx.doi.org/10.1021/jf203701f | J. Agric.Food Chem. 2012, 60, 29−35

interest. Validation experiments were carried on four separate days.

Specificity. By applying tandem mass spectrometry techniques (MS/MS), high specificity is provided. The specificity of a method refers to the extent to which a method can unambiguously detect and determine a particular analyte in a sample, without interference from other sample components, like degradants and potential matrix contaminants. According to the validation criteria, two product ions of the parent ion and one ion ratio (most intense ion over the weak ion) are needed to fulfill the identification points and confirmation requirements. To achieve higher specificity in our study, three product ions were monitored for the analyte, and two ion ratios were calculated. All ion ratios of the spiked samples were within the permitted tolerance of the EU criteria [the relative intensities of the product ions, expressed as a percentage of the intensity of the most intense product ion (base peak) when higher than 50%, must correspond to those of the calibration standards at a tolerance of 20%]. Moreover, 20 blank and 20 spiked meat and milk samples from different origins and animals were analyzed to verify the absence of endogenous interferences. All blank samples showed no interfering peaks at the same retention time as the analyte of interest. In addition, the relative retention time (RRT) between analyte and internal standard was within the required tolerance (2.5%). An example of the fulfillment of the confirmation criteria based on the ion ratio and RRT for spiked meat samples is shown in Table 2. Ion chromatograms of a meat and milk sample spiked at 0.01 ng/g of dapsone are shown in Figures 1 and 2, respectively.

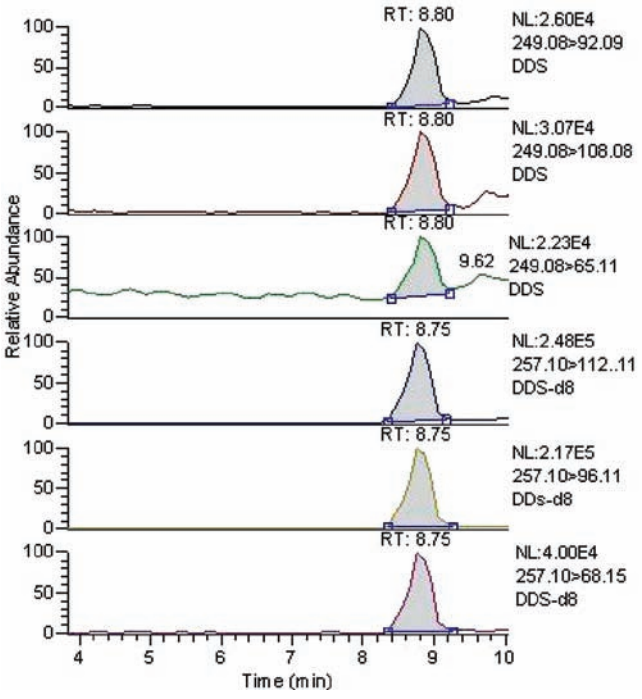

Figure 2. MRM chromatogram of a milk sample spiked with dapsone (three product ions) at a concentration of 0.01 ng/mL, with dapsone-d8 at a concentration of 0.3 ng/mL.

Matrix Effect. The interference from the matrix may have influence on the response of the analyte, increasing the background noise and causing ion suppression, which can have significant effects on the accuracy and reproducibility of the method. The matrix effect was tested by comparing the slopes of the matrix-matched calibration curves to the matrix-free calibration curves applied over a dynamic range of 0.005−1 ng/g. The p values were calculated and are shown in Table 3. The results show no significant influence ($p > 0.05$) of the meat or milk matrix on the sensitivity of the method, proving that the developed sample preparation is efficient.

Linearity. The determination of linearity and the concentration range at the method validation stage are important, because it allows the suitability of the method over the range required by the analytical specification to be established. Matrix-free calibration curves were prepared on 10 concentration levels 0.005/0.01/0.015/0.02/0.03/0.05/0.1/0.2/0.5/1 ng/g covering a dynamic range from 0.005 to1 ng/g for the analyte (Table 3). The data were evaluated by carrying out linear regression based on the ratios of detected peak areas of the analyte and adequate internal standard versus concentration. The equations and regression coefficients of the curves were calculated and found to be higher than 0.997. This dynamic range was applied for further evaluation of the validation studies. To include the recommended MRPL of 5 ng/g, 10 level calibration curves were also applied from 0.04 to 10 ng/g to evaluate the applicability of the method at the MRPL level. Linear regression values greater than 0.997 were achieved. Thus, a linearity range for dapsone was determined from 0.005 to 10 ng/g range for both bovine meat and milk samples.

Accuracy. Accuracy is a property of a single result and is influenced by both random and systematic errors. The accuracy was expressed as the mean recovery of the spiked samples in meat or milk matrix at three concentration levels: 0.02, 0.03, and 0.04 ng/g (1, 1.5, and 2 times the validation level of 0.02 ng/g). A total of 18 replicates at each concentration level over the 3 separate days, six replicates from each day, were used for the determination of the accuracy. The results of recovery studies are shown in Table 4. Recoveries were excellent ranging from 105 to 117% in meat and 101 to 108% in milk samples at all three concentration levels.

Precision. Precision is a measure of the spread of repeated measurement results and depends only on the distribution of random errors. The precision was expressed in terms of repeatability and intermediate precision (within-laboratory reproducibility). The repeatability gives an indication of the short-term variation in measurement results and is typically used to estimate the likely difference between the replicate measurement results obtained in a single batch of analysis. The intermediate precision involved making replicate measurements on 3 different days, under conditions resembling the conditions of routine use of the method. The precision results are shown in Table 4. The CV values ranged between 3.4 and 6.8% for meat samples and 2.8 and 7.6% in milk samples, indicating that the precision of the method at these low concentration levels is excellent and superior to methods cited in the literature.[9−14]

According to Commission Decision 2002/657/EC, the coefficient of variation for repeated analysis, under reproducibility conditions, should not exceed the level calculated by the Horwitz equation.[17] At these low concentration levels, the application of the Horwitz equation gives unacceptable high values and cannot be used for the evaluation of the repeatability and within-laboratory reproducibility. Thompson demonstrated that the Horwitz equation is not applicable to the lower concentration range (<120 μg/kg) as well as at high concentrations (>138 g/kg). Therefore, a complementary

Table 3. Matrix Effect Study for Calibration Curves Constructed at a Concentration Range 0.005−1 ng/g

	matrix-free calibration curve			matrix-matched calibration curve			
compd	slope	*y*-intercept	r^2	slope	*y*-intercept	r^2	*p* value
dapsone in meat	3.825	0.001	0.9981	4.201	0.002	0.9928	0.077
	3.878	0.003	0.9998	4.263	0.004	0.9924	
	3.740	0.002	0.9987	4.005	−0.006	0.9915	
	3.696	0.002	0.9992	3.820	0.003	0.9931	
dapsone in milk	4.078	0.001	0.9987	4.295	0.004	0.9937	0.185
	4.389	0.003	0.9991	4.596	−0.009	0.9911	
	4.029	0.002	0.9979	4.343	−0.005	0.9918	
	4.010	0.002	0.9981	4.101	0.005	0.9927	

Table 4. Precision and Accuracy for the Analyte on Three Separate Days Based on the Calibration Curve and the Response Factor

calibration curve		day 1		day 2		day 3		between days		
analyte	spiked (concn)	accuracy (%)	CV (%)	accuracy (%)	CV (%)	accuracy (%)	CV (%)	accuracy (%)	CV (%)	*H*(*r*)
dapsone in meat	0.02	117.2	4.6	115.4	6.8	104.4	4.0	112.3	4.0	0.28
	0.03	113.3	5.3	109.5	4.8	106.4	6.4	109.7	6.4	0.27
	0.04	108.8	3.4	105.0	4.4	106.3	3.7	106.7	3.7	0.18
dapsone in milk	0.02	106.0	4.4	108.0	3.3	107.1	7.6	107.0	7.6	0.25
	0.03	105.4	3.9	106.1	5.3	107.2	4.3	106.2	4.3	0.21
	0.04	104.3	4.0	101.3	2.8	102.7	3.9	102.8	3.9	0.18
response factor		day 1		day 2		day 3		between days		
analyte	spiked (concn)	accuracy %)	CV (%)	accuracy (%)	CV (%)	accuracy (%)	CV (%)	accuracy (%)	CV (%)	
dapsone in meat	0.02	117.1	4.5	117.4	6.6	107.1	3.5	113.9	5.1	
	0.03	112.7	5.2	110.2	4.6	104.1	6.3	109.0	4.1	
	0.04	108.0	3.4	105.1	4.3	103.7	3.6	105.6	2.1	
dapsone in milk	0.02	111.5	3.8	107.1	6.3	107.1	6.3	108.6	2.3	
	0.03	108.9	4.2	104.1	3.5	104.1	3.5	105.7	2.6	
	0.04	106.8	3.9	103.7	3.6	103.7	3.6	104.7	1.7	

model was suggested $s_H = 0.22 \times C$ for analyte concentrations $C < 120$ μg/kg and $s_H = 0.01 \times C^{0.5}$ for analyte concentrations $C > 138$ g/kg (s_H = expected standard deviation under reproducibility conditions).[18] This condition was expressed by the HORRAT(r) value, which is the ratio of the experimentally obtained values of the standard deviation and the target standard deviation calculated by the Thompson equation. As a fitness for purpose criterion, this value should be lower than 1.0.[19] The calculated $H(r)$ ratios are much lower than this criterion as shown in Table 4.

Analytical Limits. The decision limit (CCα) represents the false positive results with an error probability of 1%, and detection capability (CCβ) the false negative results with an error probability of 5%. The CCα and CCβ values were calculated based on the procedure described in the ISO 11843.[20] Matrix-matched calibration curves of six points (blank, 0.02, 0.03, 0.04, 0.06, and 0.10 ng/g) from the spiked samples were constructed on three different days, and standard deviations were calculated. CCα is equal to the concentration at the intercept plus 2.33 times the standard deviation. CCβ is equal to the concentration at the CCα plus 1.64 times the standard deviation. The estimated mean CCα and CCβ values were 0.0018 and 0.0031 ng/g in meat and milk samples, respectively (Table 1). The calculated analytical limits prove the high sensitivity of the method as they are 3 orders of magnitude lower from corresponding values reported in the literature.[9]

The applicability of the method was tested by six replicate analyses of meat and milk samples fortified at the low CCβ level and at the recommended MRPL level of 5 ng/g. For meat samples, the mean recovery was 104.7% with a CV of 4.9% at the CCβ level; at the MRPL level, the mean recovery was 108.0% with a CV of 2.3%. For milk samples, the mean recovery was 103.7% with a CV of 4.1% at the CCβ level; at the MRPL, the mean recovery was 104.8% with a CV of 1.9%. The proposed method was also tested in the analysis of 35 meat and 35 milk samples obtained from different sources. The analyte was not detected in any of the tested samples; the used quality control samples, which were spiked at the validation level, fulfilled all of the identification and confirmation criteria. Hence, the overall results were acceptable, proving that the method is capable to provide highly selective qualitative and quantitative analysis.

Uncertainty. To evaluate if a result indicates compliance or noncompliance with a specification, it is necessary to take into account the measurement uncertainty associated with the result. The expanded uncertainty U was measured and calculated by multiplying the combined standard uncertainty u with the constant k, providing an interval within a level of confidence. The combined uncertainty was estimated by taking into account the within laboratory reproducibility over the 3 separate days. On the basis of the guidelines for the implementation of decision 2002/657/EC, the coverage factor of the Gaussian distribution is proposed to 99% for a group A substance (dapsone-A6),[21] and the calculated expanded uncertainty U were 12.33% for the determination of dapsone in meat samples and 12.64% in milk samples.

dx.doi.org/10.1021/jf203701f | J. Agric.Food Chem. 2012, 60, 29−35

Table 5. Stability Test Results for the Analyte at Different Temperature Levels in Standard Solution and Matrix

	0 week	1 week		2 weeks		3 weeks		20 weeks	
storage conditions	concn	mean concn	CV (%)	mean concn	CV (%)	mean concn	CV (%)	mean concn	CV (%)
+20 °C in standard solution	5.07	5.08	3.5	5.20	5.3	5.01	4.6	4.66	5.1
+4 °C in standard solution	5.09	5.09	4.2	5.16	2.3	5.21	2.6	4.89	4.9
−20 °C in standard solution	5.11	5.12	3.1	5.25	2.1	5.21	3.3	5.31	1.8
−20 °C in meat	5.40	5.42	2.9	5.39	2.6	5.61	5.9	5.52	6.3
−20 °C in milk	5.25	5.28	3.7	5.34	3.9	5.17	4.4	5.33	4.9

Stability Tests. The stability standard solution in methanol was investigated at a dapsone concentration of 5 ng/mL. A fresh stock solution was prepared and diluted to the specified concentration and divided into sufficient aliquots. The concentration of the analyte was measured at the freshly prepared solution, and then, the test solution was divided as follows: 10 aliquots at −20 °C, 10 aliquots at +4 °C, and 10 aliquots at +20 °C.

The stability of the analyte in matrix was also evaluated. A blank meat and milk sample each was divided in five aliquots. Each aliquot was spiked at a concentration of 5 ng/g. One aliquot was measured, and the others were immediately stored at −20 °C. The storage time for both stability tests was 1, 2, 4, and 20 weeks. Changes in stability of the analyte were calculated and evaluated by the mean concentration and the CV (%) as shown in Table 5. It can be considered that the analyte in matrix is stable for at least 20 weeks at −20 °C. The standard solution stored at +20 °C was degraded, but only minor degradation occurred at +4 °C after 20 weeks. Hence, it is recommended that the working standard solutions should be stored at +4 °C for a period not longer than 20 weeks.

Response Factor (*F*). The possibility to simplify the analysis, without compromising the analytical quality, by omitting the calibration curves was studied. Predetermined response factors (*F*) were used for this approach. From the data collected from the validation study, the response factors were calculated for dapsone in meat and milk and are shown in Table 6. On the basis of these values, the concentration of all spiked samples was recalculated without applying a calibration curve. The accuracy and precision of the method are shown in Table 4. Comparison of these values with the values calculated after the analysis of a series of spiked samples (Table 3), no significant differences were found regarding precision and accuracy. Therefore, by using LC-MS/MS and a stable deuterated internal standard, it was possible to obtain equally good results by calculating the results directly from the analyte/internal standard peak area ratio and a predetermined response.

Table 6. Calculated *F* Values for the Analyte

	meat			milk		
concn (ng/g)	day 1	day 2	day 3	day 1	day 2	day 3
0.01	1.11	1.23	1.07	1.08	1.22	1.09
0.02	1.16	1.25	1.24	1.37	1.23	1.37
0.03	1.26	1.19	1.15	1.26	1.27	1.29
0.04	1.12	1.20	1.18	1.26	1.37	1.24
0.06	1.15	1.09	1.12	1.19	1.19	1.20
0.1	1.19	1.18	1.16	1.14	1.25	1.16
0.2	1.25	1.23	1.12	1.40	1.38	1.31
0.5	1.07	1.15	1.03	1.18	1.25	1.12
1.0	1.16	1.17	1.11	1.23	1.33	1.23
10	1.08	1.08	1.02	1.24	1.31	1.14
1.5	1.10	1.18	1.11	1.23	1.24	1.17
2.0	1.04	1.08	1.09	1.19	1.34	1.13
3.0	1.15	1.15	1.16	1.13	1.24	1.22
5.0	1.01	1.01	1.06	1.19	1.19	1.13
mean CV (%)	1.16	1.19	1.13	1.23	1.28	1.22
	5.9	5.5	5.4	7.0	5.0	6.8

The method is rapid and simple, as it uses an efficient extraction procedure employing automated SPE cleanup. This experimental setup avoids consumption of large solvent volumes and increases sample throughput and capacity. The APCI ionization technique was applied for the first time instead of the commonly used ESI mode. Monitoring three product ions provided sufficient selectivity for the unambiguous detection and confirmation of the analyte. The method was validated thoroughly and was proven superior to published methods by offering higher sensitivity, accuracy, and precision along with remarkable linearity. The method was further evaluated based only on the response factor resulting in equal precision and accuracy to the construction of a calibration curve. This approach greatly simplifies analyte quantification. Therefore, it can be concluded that the developed method shows good potential and is fit to be used in the frame of official control by routine laboratories.

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REFERENCES

(1) Lang, P. G. Sulfones and sulfonamides in dermatology today. *J. Am. Acad. Dermatol.* **1979**, *1*, 479−492.

(2) Jaeger, A.; Sauder, P.; Kopferschmitt, J.; Flesch, F. Dapsone (PIM 167). *IPCS, INCHEM*, 1993.

(3) Jaeger, A.; Sauder, P.; Kopferschmitt, J.; Flesch, F. Clinical features and management of poisoning due to antimalarial drugs. *Med. Toxicol.* **1987**, *2*, 242−273.

(4) Kobe, Y.; Setoguchi, D.; Kitamura, N. Dapsone-induced agranulocytosis leading to perianal abscess and death: A case report. *J. Med. Case Rep.* **2011**, *5*, 107.

(5) Meyerson, M. A.; Cohen, P. R. Dapsone-induced aplastic anaemia in a woman with bullous systemic lupus erythematosus. *Mayo Clin. Proc.* **1994**, *69*, 1159−1162.

(6) Council Regulation (EC) 37/2010, of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Off. J. Eur. Commun.* **2010**, *L15*, 1−72.

(7) CRLs view on state of the art analytical methods for national residue control plans. *CRL Guidance Paper*, 2007; 1−8.

(8) 657/2002/EC Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.* **2002**, *L221*, 8−36.
(9) Hadjigeorgiou, M.; Papachrysostomou, C.; Theodorou, Z.; Kanari, P.; Constantinou, S. Determination of dapsone in meat and milk by liquid chromatography tandem mass spectrometry. *Anal. Chim. Acta* **2009**, *637*, 220−224.
(10) Rhijn, J. A.; Lasaroms, J. J. P.; Berendsen, B. J. A.; Brinkman, U. A. T. Liquid chromatographic-tandem mass spectrometric determination of selected sulphonamides in milk. *J. Chromatogr., A* **2002**, *960*, 121−133.
(11) Suhren, G.; Heeschen, W. Detection of eight sulphonamides and dapsone in milk by a liquid chromatographic method. *Anal. Chim. Acta* **1993**, *275*, 329−333.
(12) Hela, W.; Brandtner, M.; Widek, R.; Schuh, R. Determination of sulfonamides in animal tissues using cation exchange reversed phase sorbent for sample cleanup and HPLC−DAD for detection. *Food Chem.* **2003**, *83*, 601−608.
(13) McDonald, M.; Mannion, C.; Rafter, P. A confirmatory method for the simultaneous extraction, separation, identification and quantification of tetracycline, sulphonamide, trimethoprim and dapsone residues in muscle by ultra-high-performance liquid chromatography-tandem mass spectrometry according to Commission Decision 2002/657/EC. *J. Chromatogr., A* **2009**, *1216*, 8110−8116.
(14) Peters, R. J. B.; Bolck, Y. J. C.; Rutgers, P.; Stolker, A. A. M.; Nielen, M. W. F. Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry. *J. Chromatogr., A* **2009**, *1216*, 8206−8216.
(15) Nilsson, L. B.; Eklund, G. Direct quantification in bioanalytical LC−MS/MS using internal calibration via analyte/stable isotope ratio. *J. Pharm. Biomed. Anal.* **2007**, *43*, 1094−1099.
(16) Theodoridis, G. A.; Papadoyannis, I. N. Novel Advanced Approaches in Sample Preparation and Analyte Detection for Bioanalysis. *Curr. Pharm. Anal.* **2006**, 385−404.
(17) Horwitz, W.; Albert, R. Performance characteristics of methods of analysis used for regulatory purposes. Part II. Pesticide formulations. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 718−744.
(18) Thompson, M.; Wood, R. The International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories. *J. Assoc. Off. Anal. Chem.* **1993**, *76*, 926−940.
(19) Gonzalez, A. G.; Herrador, M. A. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *TrAC, Trends Anal. Chem* **2007**, *26*, 227−238.
(20) ISO 11843-2: Capability of detection. *Methodology in the Linear Calibration Case*; International Organization of Standardization: Switzerland, 2000; pp 1−34.
(21) SANCO/2004/2726-rev 4-December 2008, Guidelines for the implementation of Decision 2002/657/EC, 2008; pp 1−10.