Determination of salicylic acid in feed using LC-MS/MS

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Abstract

Introduction: Salicylic acid is a derivative of benzoic acid and occurs in nature. The main target of this study was to develop the liquid chromatography coupled with tandem mass spectrometry technique as a method for determination of salicylic acid in feed materials and compound feed. **Material and Methods:** Salicylic acid was extracted from feed with 0.1% hydrochloric acid in methanol. Separation was achieved in 8 min in a gradient elution using 0.1% formic acid and acetonitrile. The analyte was detected using negative electrospray tandem mass spectrometry. The procedure was validated to the specifications of the European Commission Decision No. 2002/657/EC. **Results:** The validation results showed the repeatability of the method, which was evaluated at three levels (0.25, 0.5, and 1.0 mg/kg). Calibration curves for the working ranges were linear (R^2 0.9911 to 0.9936), and recoveries ranged from 98.3% to 101%. The LOD and LOQ for compound feed were 0.02 and 0.05 mg/kg, respectively. Salicylic acid was found mostly in corn, and its concentrations differed depending on whether it was young or fully grown (5.30–12.8 mg/kg and 0.13–1.01 mg/kg, respectively). **Conclusions:** A sensitive and reliable method for the determination of salicylic acid in feed and compound feed using LC-MS/MS was developed.

Keywords: salicylic acid, feed, LC-MS/MS.

Introduction

Salicylic acid (*Acidum salicylicum*) (henceforth SA) is a derivative of benzoic acid (Fig. 1) occurring in nature. It is an important signal molecule in plants; it regulates growth and development and mediates plants' response to viral and bacterial infections (1, 10). Its natural source is the white willow (*Salix alba*), mainly the roots of the tree. Plant extracts containing salicylates have been used since ancient times to control fever and pain in humans, whereas in veterinary medicine, particularly in the treatment of farm animals, the use of salicylates on a larger scale only began in the 1970s.
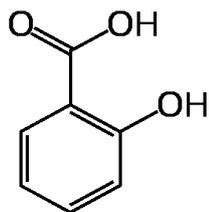

Fig. 1. Structure of salicylic acid

As SA is widely distributed throughout the plant kingdom, its systemic presence in humans may arise from the consumption of plant-based foods. Salicylates in high doses may be dangerous. A certain percentage of the population is hypersensitive to this compound, and these persons are advised to avoid products containing SA. The implications of hypersensitivity to salicylates include Widal syndrome, aspirin-induced asthma or asthma with hypersensitivity to aspirin, Reye's syndrome (encephalopathy and fatty degeneration of internal organs), and angioneurotic oedema or urticaria (2, 5).

The content of salicylates decreases as fruit ripens; also cooked foods as a rule contain less salicylates than fresh and dried ones. Vegetables have a large range in their content of salicylates, from 0 to 60 mg/kg (15), and besides vegetables, herbs, and spices contain large amounts of salicylates. When contained in food this compound is released in the digestive system, providing a source of SA (8).

It is assumed that products of animal origin (meat, fish, eggs, milk, and dairy products) do not contain salicylates or contain only trace quantities of them (4, 8). However, the administration of drugs to animals may

result in their presence in tissues and animal origin products, which may pose a potential risk to consumers. Moreover, naturally occurring SA can also be present in feed, thus being a source of additional animal and human exposure to this compound.

Using different analytical techniques and complicated methods, SA has been detected in many different commodities (vegetables, fruits, grains, seeds, nuts, herbs, spices, and other food) (11, 14, 15, 17, 18). These methods are often time-consuming and require large volumes of potentially harmful solvents (especially dichloromethane) and large sample quantities.

It being that many plants are materials for the production of feed for animals, it was decided to develop a sensitive and accurate method for the determination of SA in feed materials and compound feed. The obtaining of first data on the concentrations of SA in animal feeds was one goal, and another was the further use of the developed method to study the transfer of salicylates to food of animal origin.

Material and Methods

Chemicals and reagents. Methanol was purchased from P.O.Ch. (Poland). Acetonitrile was from J.T. Baker (USA). Hydrochloric acid (35%–38%) was bought from Chempur (Poland) and formic acid from Sigma-Aldrich (Germany). SA and SA–d4 were obtained from Sigma-Aldrich (USA). Water was purified through a Milli-Q Plus system from Millipore (USA) and PTFE syringe filters (0.2 μm, 25 mm) were supplied by Restek (USA).

Preparation of standard solutions. The stock standard solutions (1,000 μg/mL) of SA and SA–d4 (internal standard) were prepared by weighing the appropriate amount of the substances and dissolving in acetonitrile. Both stock solutions were kept at below −18°C for 12 months. Working standard solutions were prepared by dilution of appropriate amounts of standard solutions with acetonitrile and kept at below −18°C for three months.

Materials. Samples of commercially available feeds for poultry, pigs, and cattle were collected. Grains (wheat, barley, triticale, corn) were obtained from a farm cultivating these plants. Ground paprika was purchased in local supermarkets.

LC-MS/MS. The LC-MS/MS system consisted of a Shimadzu Nexera X2 HPLC liquid chromatograph (Shimadzu, Japan) coupled with an 8050 triple quadrupole detector (Shimadzu, Japan), controlled by LabSolution 5.60 SP2 software (Shimadzu, Japan). The separation was performed using a Poroshell 120 EC-C18 analytical column (2.1 × 100 mm, 2.7 μm) (Agilent, USA) with a C18 guard cartridge operated at 30°C. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B); the gradients were 5% B (0 to 1.0 min), 90 % B (2.5 to 5.0 min), and 5% B (6.0 to 8 min); the flow rate was 350 μL/min; and the injection volume was

2 μL. The experiments were conducted in the negative electrospray mode. Ion-source parameters were as follow: nebulising gas flow was set to 2 L/min and heating gas and drying gas flows were set to 10 L/min. The interface temperature was 300°C, temperature of desolvation line was set to 240°C, and heat block temperature was 400°C. SA was quantified using the selected reactions monitoring (SRM) mode (137→93, 137→65).

Sample preparation. Feed samples (2.5 g) were weighed into 50 mL polypropylene centrifuge tubes. Next, 25 mL of 0.1% hydrochloric acid in methanol was added, and the samples were vortexed. Then the samples were transferred to an ultrasonic bath for 15 min. Extract was obtained using a shaker mixer (200 rpm, 30 min) and centrifuged (4,500 x g, 20°C, 15 min). A total of 500 μL of extract was placed in a glass tube for evaporation (N$_2$, 45°C). The dry residues were reconstituted with 500 μL of 50% acetonitrile, transferred to 1.5 mL Eppendorf tubes and centrifuged at 14,500 rpm for 15 min. Finally, the aliquot of 100 μL of supernatant was transferred to an HPLC vial, 10 μL of internal standard and 390 μL of 50% acetonitrile were added, and the resulting solution was analysed using the LC-MS/MS technique.

Quantification of SA in samples. For quantitative analysis, the standard addition approach was used. For each sample, a calibration curve was separately prepared and analysed. Before instrumental analysis, seven 100 μL aliquots of the supernatant were taken, to each one an aliquot of 10 μl of internal standard (SA-d4) 0.5 μg/mL was added, and the extracts were fortified with appropriate quantities of working standard solution and refilled to 500 μL with 50% acetonitrile. Analyte concentrations in the matrix calibration curve were 0, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 mg/kg.

Method validation. To prove fitness of purpose of the developed method, validation was performed according to the European Commission Decision No. 2002/657/EC (3). The linearity of response for concentrations of SA was verified as a part of the validation against a matrix-matched calibration curve prepared at 0.1, 0.25, 0.5, 1.0, and 2.0 mg/kg for corn and 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 mg/kg for compound feed. The repeatability and reproducibility were calculated from the results of three series of samples in six replicates fortified at three levels of SA in feed. The limit of detection (LOD) of the method was evaluated on the basis of a signal-to-noise ratio of three. The limit of quantification (LOQ) was the lowest point on the different calibration curves for corn and compound feed.

Results

The sample extraction gave satisfactory results, and SA was determined with sufficient sensitivity. Because the presence of SA was detected in almost every sample,

the standard addition method was used for quantification. Chromatograms of wheat and corn samples and the same samples with a standard addition at 0.5 mg/kg are presented in Fig. 2.

Results of method validation for corn and compound feed are presented in Table 1. Good linearity was obtained. Recoveries of the described method for corn and compound feed were in the range of 97.8%–101% and 95.3%–105%, respectively. The repeatability and reproducibility values were acceptable (below 15% in all cases). The limit of detection (LOD)

and limit of quantification (LOQ) values were also determined and proved to be fit for purpose.

Several types of compound feeds destined for laying hens, pigs, and cattle, and various feed materials (wheat, barley, triticale, ground paprika, young corn, fully-grown corn) were tested with the developed method. For the compound feed, depending on the percentage composition of individual feedstuffs, results in the range from <LOQ to 0.48 mg/kg were obtained. The grains contained negligible amounts of SA. The highest concentrations were detected in ground paprika and in corn. The results are shown in Table 2.
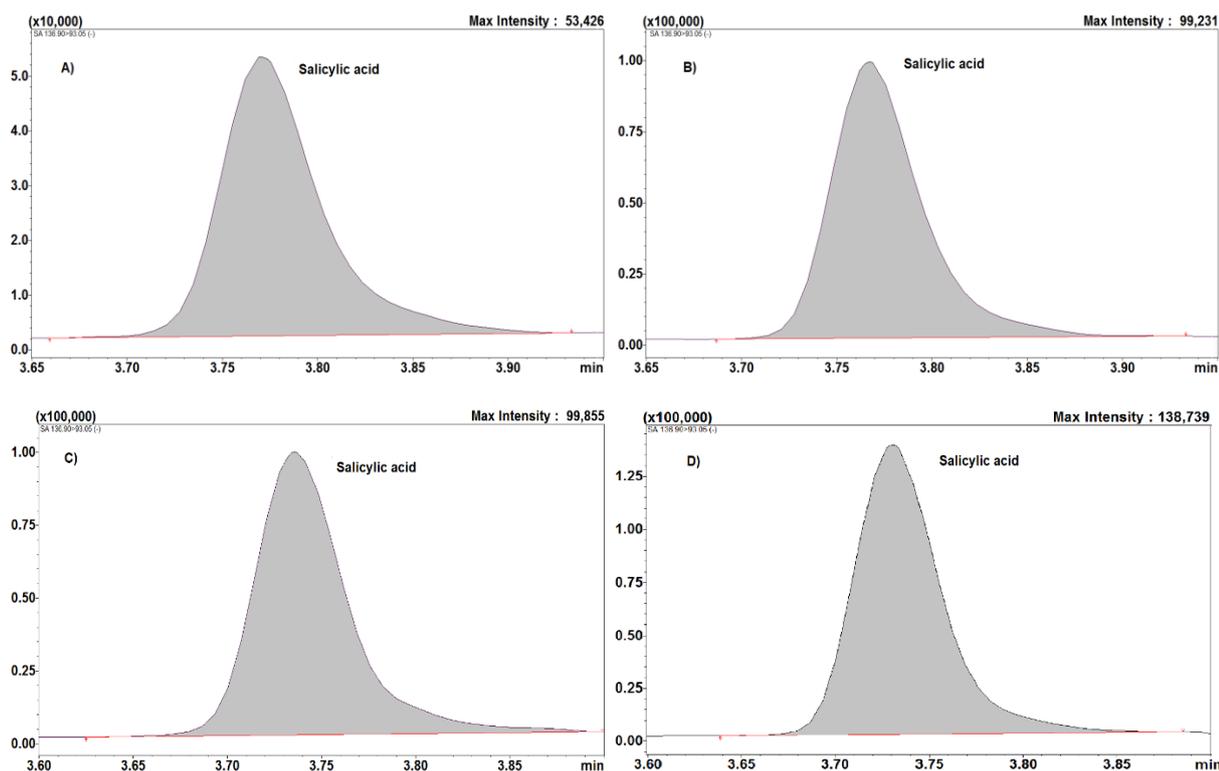

Fig. 2. Chromatograms of SA in feed: wheat (A), wheat with a standard addition at the level of 0.5 mg/kg (B), corn (C), and corn with a standard addition at 0.5 mg/kg (D)

Table 1. Results of method validation for corn and compound feeds

Validation parameter	Material					
	Corn			Compound feed		
Linearity (R^2)	0.9936			0.9911		
Limit of detection, LOD (mg/kg)	0.05			0.02		
Limit of quantification, LOQ (mg/kg)	0.1			0.05		
Concentration (mg/kg)	0.25	0.5	1.0	0.25	0.5	1.0
Repeatability, CV (%)	8.74	7.21	6.93	7.28	11.3	8.06
Reproducibility, CV (%)	9.63	11.8	7.46	8.15	14.7	9.35
Recovery (%)	101	98.3	97.8	105	100	95.3

Table 2. Results of determination of salicylic acid in feeds

Type of feed	Concentration (mg/kg)
Wheat (n=3)	0.05–0.08
Barley (n=3)	<LOQ
Triticale (n=3)	<LOQ
Ground paprika (n=3)	0.58–1.87
Young corn (n=3)	5.30–12.8
Fully grown corn (n=3)	0.13–1.01
Compound feed (n=50)	<LOQ–0.48

n – number of tested samples

Discussion

In the method developed here for SA determination, the technique of liquid chromatography with mass spectrometry was applied, while the available studies on SA determination describe the use of gas chromatography with mass spectrometry (14), high-performance liquid chromatography with UV (15), electrochemical (18) or fluorescence detection (11, 16, 17), and spectrofluorimetry (12). Despite the use of less selective analytical techniques, it was possible to obtain low detection limits. For example, the LOD of the method of Venema *et al.* (17) was 0.02 mg/kg for fresh food, which is similar to our method (0.02 mg/kg for feed (wheat, barley, and triticale) and 0.05 mg/kg for corn).

SA was extracted from samples with 0.1% hydrochloric acid in methanol which provided similar recoveries as previously reported using acidified acetonitrile (17). The time of analysis of the samples until the chromatographic analysis was short, which allowed many samples to be analysed per workday. In the presented method we detected free SA, while other authors (15, 17, 18) often performed overnight alkaline hydrolysis to also determine the fraction bound to the matrix. Such an approach was tested during the method optimisation work, however, after hydrolysis the extracts were too cloudy to be injected into the LC-MS system. According to Venema *et al.* (17), the ratio of free to total SA was matrix-dependent but in most cases it was around 0.8. Taking these results into account, we limited the scope of our method to free SA, assuming it was still a good prognostic of total SA content.

In analytical methods based on electrospray mass spectrometric detection so-called ion suppression may occur as a result of interference with sample matrix constituents (7). Because of this phenomenon, quantitation should be performed using matrix-matched calibration curves, more resembling the analytical samples than the pure reference standard solutions. Sometimes, however, when the analytes occur naturally in the tested material, no blank sample can be found. Such was the case in this application and therefore we used the standard addition approach, which provided reliable results in a relatively fast and labour-efficient manner.

Feed is a complicated and highly heterogeneous sample matrix and the performance of the LC-MS/MS based method can be influenced by the type of sample (9). Taking into consideration labour and costs, it was not possible to perform full validation for all feed types. Therefore, it was decided to restrict it to the most important matrices: corn, as the one expected to contain the highest concentrations of SA and compound feed and simultaneously the most complex sample and the actual source of SA for animals. In both types of sample, the quantitative performance of the method was comparable. The obtained sensitivity (LOD/ LOQ) was better for the compound feed which was related both to the chemical composition of samples and the higher background for corn. The characteristics of the method for the compound feed were assumed to be applicable for the grains and paprika samples.

The developed method was used for analysis of several feed materials and compound feeds. As seen in Table 2, SA was determined in wheat, corn, ground paprika, and some compound feed samples. The obtained results are comparable to those obtained by other authors in recent years (6, 11, 17). In a study by Kęszycka *et al.* (6) who analysed different Polish foods, the levels of SA were much higher in corn (0.79 mg/kg) than in wheat (below LOD to 0.014 mg/kg). The concentrations detected previously in spices reached 28.6 mg/kg (14, 17, 18), and were higher than the range of 0.58–1.87 mg/kg determined in this study.

The most comprehensive study on the occurrence of SA in foods was performed by Swain *et al.* (15) in 1985. The concentrations of SA determined by these authors were higher than cited above: 1.0–78 mg/kg in fruits, 2.8–2180 mg/kg in spices, and 4.3 mg/kg in corn, with even ten-fold variations within the commodity groups. Some authors (17) have already discussed these results suggesting that the mode of detection (ultraviolet spectrophotometry at 235 nm wavelength) was not selective enough and that the authors overestimated SA levels in food. Swain and her collaborators explained their results by the effective extraction protocol they had applied. This is, however, doubtful because similar, if not identical sample preparation was used by both Kęszycka *et al.* (6) and Venema *et al.* (17), who obtained much lower levels of SA in food. In addition, Swain and her fellow authors did not provide any validation parameters to confirm the reliability of their method.

In our study the most interesting case was corn. The SA contents determined in young and fully-grown corn were 5.30–12.8 mg/kg and 0.13–1.01 mg/kg, respectively. As previously stated, the levels of salicylates decrease as the fruits ripen (13); the same may apply to grains. Other investigators found SA at a concentration in the range of 0–0.82 mg/kg (11, 12, 14, 15). However, the SA values obtained by them were given for sweet or processed corn, and none of them examined young corn in early development with the cob.

As for the compound feed, there is no literature data on the occurrence of SA. All the samples tested in this study contained corn, but, unfortunately, the percentage composition of individual feeds was not precisely described by the feed producer. Still, it can be concluded that SA present in compound feeds comes mainly from the presence of relatively large amounts of corn.

In conclusion, the validation data demonstrate that the chromatographic method for the determination of SA in compound feed and feed materials is reliable and reproducible. Furthermore, the described assay offers a number of advantages in terms of simplicity, reduced analysis time, consumption of organic solvents, and cost of analysis. The concentration of SA in tested materials was comparable to those obtained by other authors.

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