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A Simple and Robust Protocol for fast RP-HPLC Determination of Salicylates in Foods

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Abstract In this methodological report, we present a simple, versatile, and reliable procedure for quantitative determination of free and conjugated forms of salicylic acid (SA) in various food products using reversed phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection. The presented sample preparation protocol is considerably simplified in comparison to procedures applied previously and is based on three simple and fast extraction steps providing a supreme tool for large-scale routine assays. The limits of detection were approximately 0.021 $\mu g\ g^{-1}$ of dried weight for spices, lyophilized fruits, or vegetables, and 0.001 $\mu g\ ml^{-1}$ for beverages. The recoveries of the spiked SA were in the range of 87.6 to 96.6 % for all studied products. Applicability of the method was verified by the analysis of salicylate content in a wide range of products including spices (curry, oregano, red pepper), beverages (beer, brewed tea, milk, wine), lyophilized fruits (apricot, strawberry, watermelon), and vegetables (cucumber, tomato).

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Introduction

Salicylates are a group of chemical compounds comprising free salicylic acid (SA) and its various derivatives, mainly glycosides and esters. They occur naturally in many food products, including fruits, vegetables, beverages, herbs, and spices (Swain et al. 1985, Scotter et al., 2007, Wood et al. 2011). Plant salicylates act as protection agents against harmful insects, fungi, and bacteria. Synthetically produced salicylates are found in over-the-counter medications such as aspirin, as well as numerous prescribed medications, cosmetics, and preservatives.

Several studies indicate that increased intake of salicylates from natural sources may be beneficial (Duthie and Wood 2011). However, there is a group of adults and children for which even a small dose of salicylates can cause serious health problems. Consumption of salicylates by these patients may cause the symptoms such as asthma, nasal polyps, urticaria, or angioedma. Patients with salicylates hypersensitivity also experienced abdominal pain, diarrhea, headache, and constitutional symptoms (Swain 1988, Morwood et al. 2005). One of the factors which may beneficially reduce the symptoms in salicylate-sensitive individuals is a proper, low-salicylate diet (Swain 1988, Cunningham 2010). For this purpose, the patients, as well as dietitians planning salicylate-restricted diets, should obtain the detailed information regarding salicylate content in foods. Unfortunately, these data are rather incomplete and often contradictory (Swain 1988, Janssen et al. 1996, Wood et al. 2011), which might be associated with the lack of the relatively cheap, fast, and simple method that allows the routine determination of salicylates in a wide range of food products. Most of the existing methods are dedicated to analyze cosmetics, pharmaceuticals, plasma, or urine (Mikami et al. 2002, Torriero et al. 2004, Vree et al. 1994, Kees and Jehnich 1996, Salinas et al. 1990), where due to the

uncomplicated matrix with a low content of interfering substances and rather large content of an analyte, essential sample preparation process is not required. In turn, only a limited number of the protocols have been developed for the determination of free SA or its derivatives in foods. Moreover, these protocols included complicated and cumbersome multi-step sample processing (Swain et al. 1985, Venema et al. 1996), required rather sophisticated and expensive equipment (Scotter et al. 2007, Gruz et al. 2008), or were limited to one group of food products (Paterson et al. 2006, Irakli et al. 2012).

Therefore, the purpose of this study was to develop a simple, versatile, and fast method for the determination of salicylates in a large number of samples from various food products. Taking into consideration that SA is present in foods either in the free or bound form, and presumably the physiological effects of these two forms may be different (Hare et al. 2003), it was aimed to elaborate the method in such a manner that it allows the determination of free SA content and the overall amount of salicylates under possibly uniform sample preparation conditions. Especially, that up to date two separate protocols utilizing different extraction conditions were applied for the determination of free SA and free + bound SA content in foods (Venema et al. 1996, Irakli et al. 2012). In our opinion, such an approach may lead to some data inconsistencies due to possible differences in the free SA extraction levels when two different solvents are used. It is worth to note that most of the recently developed protocols enhanced only the methodology of free SA content determination (Scotter et al. 2007, Gruz et al. 2008, de Souza et al. 2014).

Materials and Methods

Reagents

Salicylic acid was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). HPLC-grade acetonitrile and ethyl acetate were purchased from Roth (Karlsruhe, Germany). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from POCH SA (Gliwice, Poland). All aqueous solution were prepared using ultra-pure Milli-Q water. HPLC eluents were filtrated prior use.

Initial Processing of Food Products

Fresh fruits and vegetables were freeze-dried with a freeze dryer Labconco 2.5 at a temperature of −40 °C and under pressure of 0.100 mBa. The freeze-dried samples were pulverized to a fine powder using a laboratory mill A-11. The pulverized fruits and vegetables were placed in plastic tubes and stored at −80 °C to prevent the loss of salicylates. Oregano was pulverized to a fine powder with mortar and pestle before

extraction. Tea was prepared as infusion (2 g of tea brewed for 2 min in 200 mL of boiling water). Curry, red pepper, beer, milk, and wine were directly subjected for extraction.

Sample Preparation Procedure

Sixty milligram weights of dry food products (i.e., spices, lyophilized fruits and vegetables) were placed in 2.0-mL eppendorf tubes, and to each tube, 1.5 mL of 0.4 M K_2HPO_4 was added. For beverages (i.e., beer, brewed tea, milk, or wine), 1 mL of each product was mixed with 0.5 mL of 1.2 M K_2HPO_4. All the samples were then vigorously vortexed until fine mixture was obtained (approximately 30–180 s depending on product) and subsequently incubated for 15 min at 70 °C. After incubation, the samples were additionally vortexed for approximately 30 s, and centrifuged at 16,000×g for 10 min. Subsequently, 1 mL of the supernatants were taken up and placed in the 2-mL safe-lock eppendorf tubes containing 150 μL of 10 M HCl. Obtained acidified supernatants were then either directly extracted with ethyl acetate (*free SA determination*) or subjected to hydrolysis by incubation at 95 °C for 90 min and cooled down to a room temperature prior to ethyl acetate extraction (*free + bound SA determination*). Ethyl acetate extractions were performed as follows: 850 μL portions of ethyl acetate were added to the acidified supernatants, the mixtures were then extensively vortexed for 60 s and centrifuged at 16,000×g for 10 min. Next, 600-μL portions of the upper organic phase were removed and placed in the 2-mL safe-lock eppendorf tubes containing 600 μL of 0.2 M phosphate buffer (pH 7.8; prepared by dissolving 1.25 g of KH_2PO_4 and 15.82 g K_2HPO_4 in 0.5 L of H_2O). The samples were then vigorously vortexed for about 60 s and centrifuged at 16,000×g for 1 min. Finally, 200 μL portions of the lower aqueous phase were removed and placed either in HPLC vials for direct analysis, or in a new eppendorf tube and stored at −80 °C. Schematic representation of the sample preparation process is shown in Fig. 1.

Analytical Equipment and Chromatographic Conditions

The HPLC system consisted of a binary pump (Model 1525, Waters Corporation, Milford, MA, USA), a fluorimetric detector (Model 474, Waters), an autosampler (Model 717plus, Waters), and a personal computer with Breeze data acquisition and integration software (Waters). Chromatographic separations were carried out at room temperature on a C8 column (Symmetry 4.6 × 150 mm, 5 μm, Waters) guarded by a C8 precolumn (Symmetry 3.9 × 20 mm, 5 μm, Waters) using a linear gradient elution. Sixty millimolar phosphate buffer pH 6.8 (prepared by dissolving 4.08 g of KH_2PO_4 and 5.22 g of K_2HPO_4 in 1 L of H_2O) and 80 %:20 % (*v/v*) acetonitrile:H_2O were used as eluent A and eluent B, respectively. The flow rate of the mobile phase was 1 mL min^{-1}. The mobile phase started
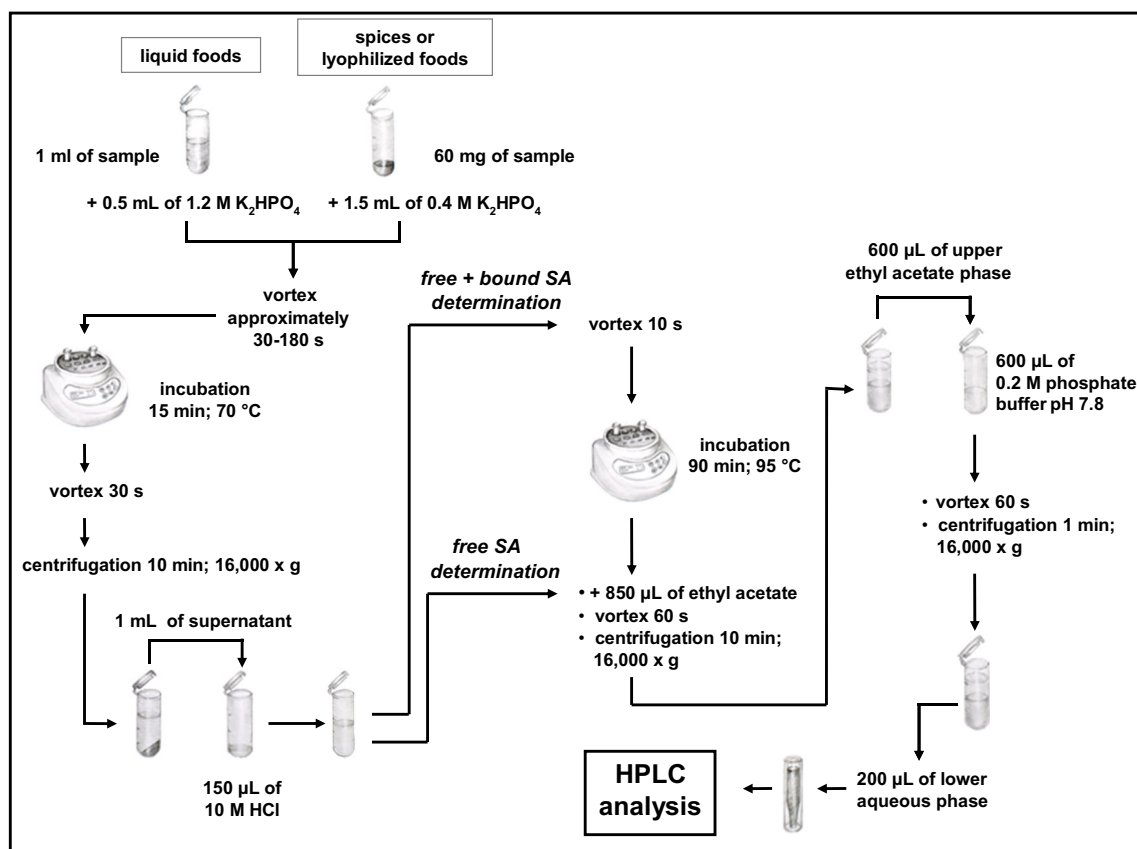
Fig. 1 Schematic representation of the sample preparation procedure

with eluent A: eluent B at 90 %:10 %, and varied to 70 %:30 %, 0 %:100 % and 90 %:10 % in 10, 13, and 22 min, respectively. The total run time was 27 min. The fluorimetric detector was set to excitation and emission wavelengths of 295 and 405 nm, respectively. The injection volumes were 20 μL. The content of SA in foods was quantified based on the prepared calibration curve.

Calibration Curve, Limits of Detection, and Quantification

A set ($n = 14$) of working solutions, containing 3.5, 10, 25, 37.5, 50, 62.5, 125, 250, 500, 1250, 2500, 5000, 7500, and 10,000 ng mL^{-1} of SA, were prepared in triplicate in 0.2 M phosphate buffer (pH 7.8) and analyzed. The injection volumes were 20 μL. Calibration curve was prepared by least squares linear regression analysis of the peak area versus amount of the injected SA. The limits of SA detection (LOD) and quantification (LOQ) were determined from chromatograms based on signal-to-noise ratio of 3 and 10, respectively. Using this approach, the lowest detectable and quantifiable amounts of the SA in the injection volume of 20 μL were estimated to be 0.02 and 0.07 ng, respectively. These values were subsequently recalculated and expressed in micrograms per gram of the dry weight for spices, lyophilized

fruits and vegetables, and in micrograms per milliliter for beverages. The recalculations were based on the following formulas:

$$LOD, LOQ = \left[\frac{\mu g}{\text{g of dry weight}}\right]$$

$$= \left[\frac{ng \times 30^a \times 850^b \times 1.5^b \times 1000^d}{600^b \times 1000^c \times 60^d}\right]$$

$$= ng \times 1.06$$

$$LOD, LOQ = \left[\frac{\mu g}{ml}\right] = \left[\frac{ng \times 30^a \times 850^b \times 1.5^b}{600^b \times 1000^c}\right]$$

$$= ng \times 0.06$$

where the individual coefficients in the equations are related to the: [a] injection volume of 20 μL out of 600 μL, [b] volume correction factor for extraction steps, [c] units conversion from ng to μg, and [d] initial sample weight.

Recovery Studies

Recovery analysis was carried out by spiking each studied food product with SA standard at two different levels corresponding to 100 % of *free SA* and 100 % of *free + bound SA*
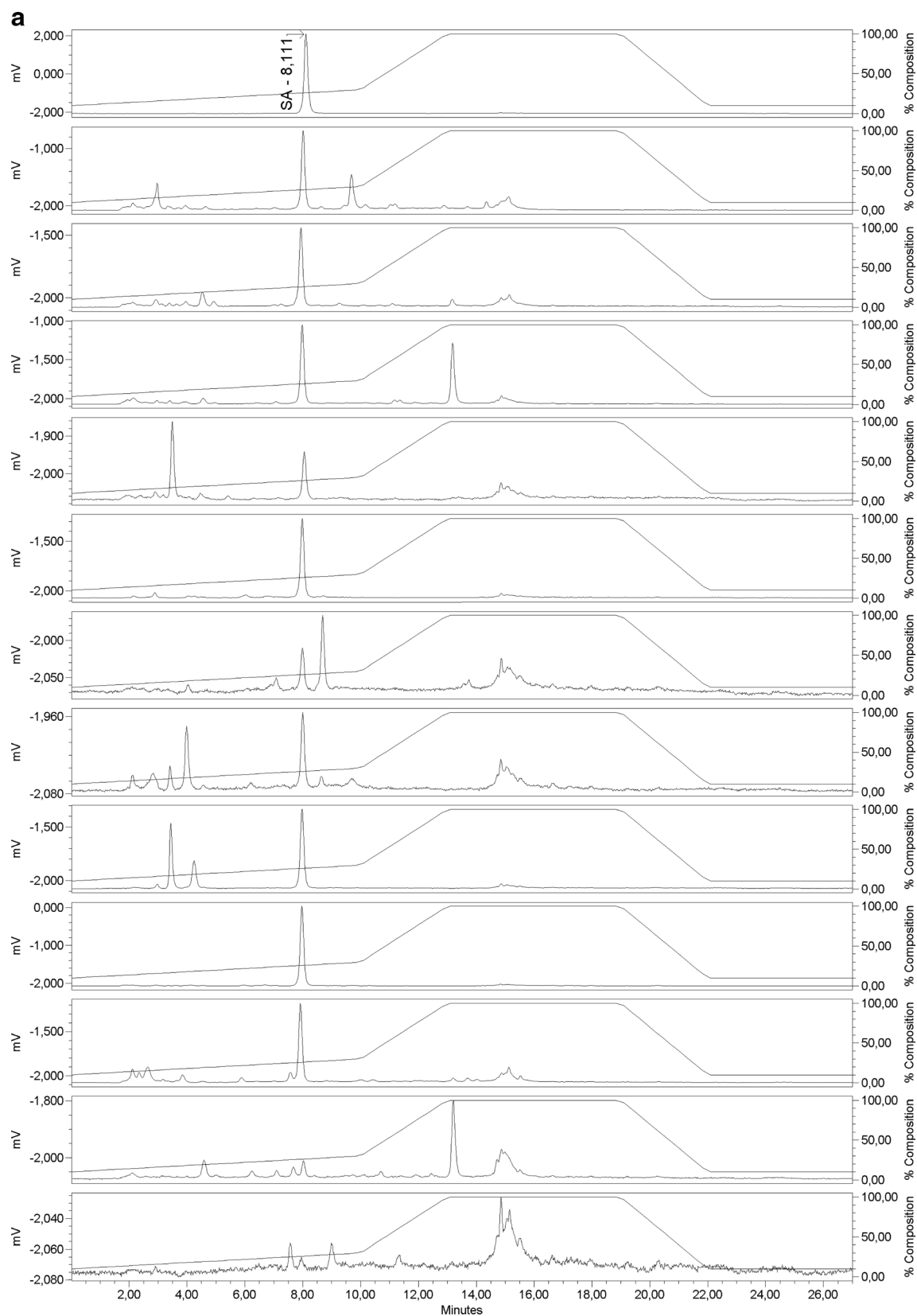
Fig. 2 Representative chromatograms obtained by RP-HPLC determination of *free SA* (**a**) and *free + bound SA* (**b**) content in various foods. Chromatogram on the top shows SA standard (1.25 μg ml^{-1}) analysis. Chromatograms from *top* to *bottom* show the analysis of samples prepared from oregano, curry, red pepper, strawberry, watermelon, apricot, cucumber, tomato, brewed tea, wine, beer, and milk. *Solid line* represents the gradient profile. All chromatograms were normalized to the highest peak
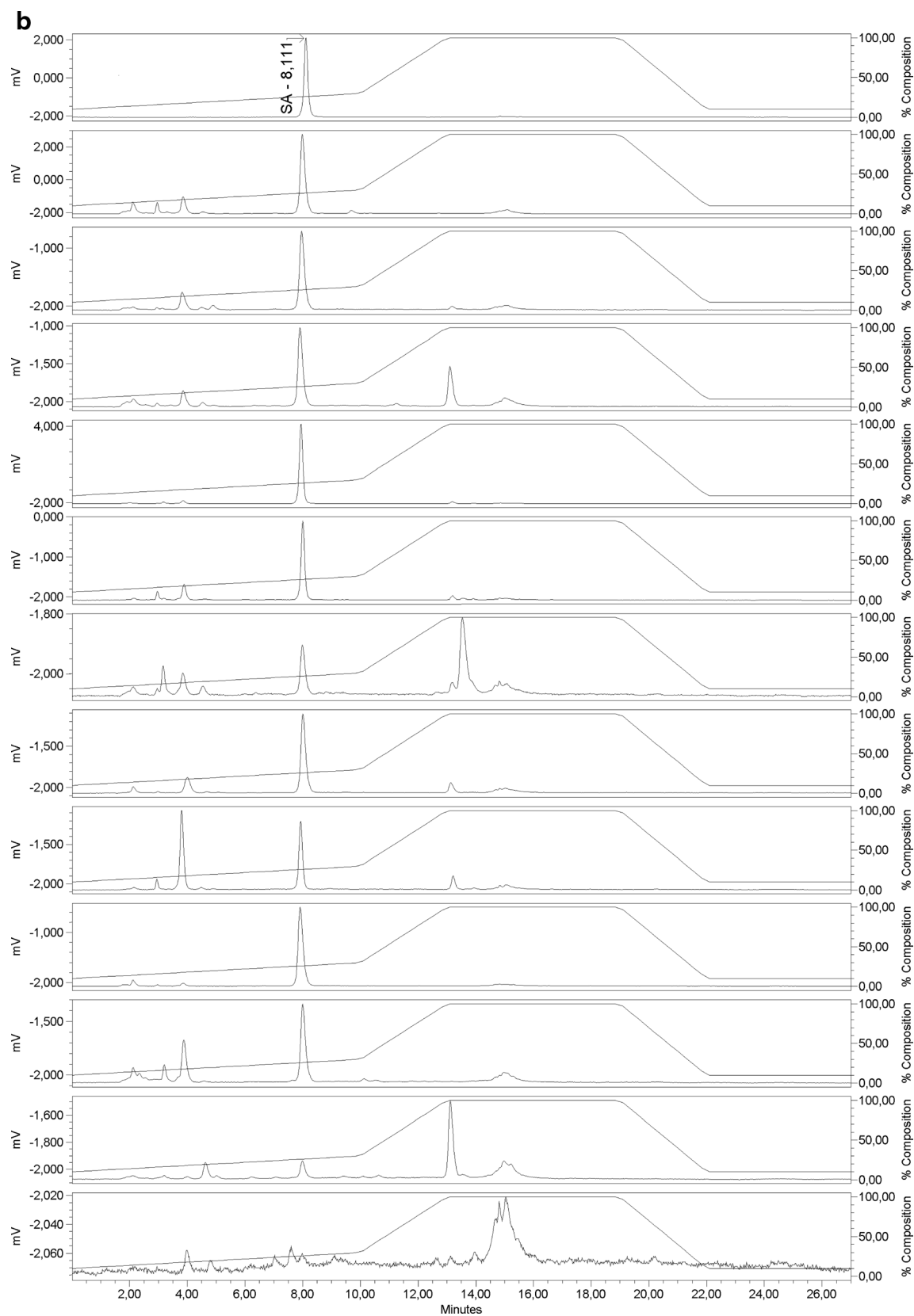
Fig. 2 (continued)

Table 1 Precision of the method in terms of intra-day and inter-day variability

Product	Intra-day variation				Inter-day variation			
	Free SA determination		Free + bound SA determination		Free SA determination		Free + bound SA determination	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Curry	3.29 ± 0.14	4.26	9.73 ± 0.39	4.01	3.33 ± 0.15	4.50	9.72 ± 0.38	3.91
Strawberry	0.71 ± 0.03	4.23	33.82 ± 1.41	4.17	0.73 ± 0.04	5.48	33.47 ± 1.56	4.66
Tomato	3.79 ± 0.17	4.49	4.74 ± 0.15	3.16	3.80 ± 0.16	4.21	4.69 ± 0.15	3.20
Brewed Tea	0.67 ± 0.02	2.99	0.70 ± 0.04	5.71	0.69 ± 0.03	4.35	0.72 ± 0.04	5.56

SA content is expressed in $\mu g\ g^{-1}$ of dry weight for curry, strawberry, and tomato, and in $\mu g\ ml^{-1}$ for brewed tea. Intra-day and inter-day variations are expressed as the relative standard deviations (RSD) of results obtained on day 1 and over 3 days, respectively

amounts found in the same non-spiked food sample. For milk sample, where *free SA* and *free + bound SA* content was below quantification limit, the fortification level was 20 ng. Subsequently, spiked samples were processed in the identical way as non-spiked samples according to the sample preparation procedure described above. Recovery (%) was calculated by comparison of SA content in spiked and non-spiked samples.

Precision of the Method

The precision of the method was evaluated in terms of intra-day and inter-day variability. Measurements were performed on curry, brewed tea, tomato and strawberry samples. In each of these products, *free SA* and *free + bound SA* content was

analyzed successively four times per day during 3 consecutive days. Intra-day and inter-day precisions were expressed as the relative standard deviations (RSD) of results from repeated measurements.

Results and Discussion

In order to substantially simplify and unify the methodology of salicylate determination in foods, we have developed an efficient and versatile protocol utilizing RP-HPLC with fluorescence detection. The established sample preparation procedure is characterized by uniform extraction conditions for *free SA* and *free + bound SA* determination in both lyophilized and liquid foods, lack of sample drying step and high

Table 2 Recovery studies and the salicylates content in the tested food products

Product	SA content[a]		Recovery studies[b]		Limits of detection and quantification[c]	
	Free	Free + bound	Free SA determination	Free + bound SA determination	LOD	LOQ
Oregano	7.69 ± 0.22	35.61 ± 1.37	91.4 ± 3.18	89.1 ± 2.55	0.021	0.074
Curry	3.34 ± 0.10	9.78 ± 0.20	93.8 ± 3.65	91.7 ± 1.60		
Red pepper	5.47 ± 0.17	7.72 ± 0.18	95.3 ± 2.79	88.8 ± 3.52		
Strawberry	0.73 ± 0.03	34.04 ± 0.99	95.8 ± 2.47	94.4 ± 3.54		
Watermelon	4.37 ± 0.12	10.62 ± 0.14	92.7 ± 3.45	93.1 ± 3.16		
Apricot	0.31 ± 0.01	1.18 ± 0.05	94.2 ± 4.07	87.6 ± 4.56		
Cucumber	0.65 ± 0.02	6.71 ± 0.20	88.2 ± 2.76	91.7 ± 3.39		
Tomato	3.94 ± 0.11	4.67 ± 0.08	92.1 ± 3.38	96.6 ± 4.12		
Brewed Tea	0.68 ± 0.01	0.70 ± 0.02	93.3 ± 2.19	91.0 ± 3.37	0.001	0.004
Wine	0.29 ± 0.01	0.32 ± 0.01	94.1 ± 3.18	89.1 ± 2.14		
Beer	0.02 ± 0.00	0.05 ± 0.00	89.6 ± 3.52	91.5 ± 2.98		
Milk	<LOQ	<LOQ	90.2 ± 2.93	88.0 ± 4.18		

[a] SA content is expressed in $\mu g\ g^{-1}$ of dry weight for spices and lyophilized foods, and in $\mu g\ ml^{-1}$ for beverages. The results are the mean ± SD of four replicates

[b] The results are the mean ± SD of four replicates

[c] LOD and LOQ values are expressed in $\mu g\ g^{-1}$ of dry weight for spices and lyophilized foods, and in $\mu g\ ml^{-1}$ for beverages

compactness, i.e., all sample preparation steps can be accomplished in the eppendorf tubes in a limited time (see Fig. 1). The sample preparation procedure is based on three simple extraction steps. In the first stage, SA and its derivatives are extracted from foods under optimized conditions using relatively concentrated (0.4 M) K_2HPO_4. We have found that the salicylate extraction yield increased together with increasing ionic strength and pH of the extraction buffer, although very alkaline buffers could not be applied due to hydrolysis of SA derivatives (data not shown). 0.4 M K_2HPO_4 solution was characterized by sufficiently high pH and ionic strength to exhaustively extract salicylates, buffered against pH changes toward the acidic direction, and also prevented potential pH shift to very alkaline region (phosphate pK_{a3} is ≈12.5). Two further steps of sample preparation, namely ethyl acetate extraction and back extraction to the phosphate buffer, took advantage of the physicochemical properties of SA. pKa of SA carboxyl group is about 2.97, and therefore by varying the pH of the solution, SA partition coefficient between aqueous and organic phase changes significantly. In our opinion, this extraction-re-extraction steps serve an excellent alternative to the commonly applied laborious and time consuming sample drying step (Swain et al. 1985, Venema et al. 1996, Scotter et al. 2007). Especially, that due to the volatility of SA, additional precautions are necessary during sample drying (Verberne et al. 2002). Moreover, applied back extraction buffer possesses not only sufficiently high concentration and pH to thoroughly re-extract SA from ethyl acetate layer, but was also found to be a suitable injection solvent. The developed sample preparation procedure allowed to obtain adequate samples purity with low level of interferences for all tested food products comprising spices (curry, oregano, red pepper), beverages (beer, brewed tea, milk, wine), lyophilized fruits (apricot, strawberry, watermelon), and vegetables (cucumber, tomato) (Fig. 2a, b). Under established and optimized chromatographic conditions, the retention time of SA was approximately 8.1 min. The dependence of the detector response on the amount of injected SA was found to be linear in the range from 0.07 to 200 ng. The resulting calibration curve ($y = 1817.1x − 168.5$) was characterized by a high determination coefficient ($R^2 = 0.9999$). Precision of the method was assessed by determining intra-day and inter-day variability of *free SA* and *free + bound SA* content in four selected food products representing lyophilized fruits and vegetables (strawberry, tomato), spices (curry), and beverages (brewed tea). The RSD values of *free SA* and *free + bound SA* content for both intra-day and inter-day precisions were between 2.99 and 5.71 (Table 1). These results indicate that the method is characterized by a satisfactory precision, irrespective of matrix type in which salicylate content is determined. The recovery rates of SA, analyzed for all 12 studied products, were in the range of 88.2 to 95.8 % for *free SA* determination method and in the range of 87.6 to 96.6 % for *free + bound SA*

determination method (Table 2). The LOD values were approximately 0.021 $\mu g\ g^{-1}$ of dried weight for spices, lyophilized fruits and vegetables, and 0.001 $\mu g\ ml^{-1}$ for beverages, while the LOQ values were approximately 0.074 $\mu g\ g^{-1}$ of dried weight for spices, lyophilized fruits, and vegetables, and 0.004 $\mu g\ ml^{-1}$ for beverages (Table 2). The obtained recovery ranges and LOD values are similar with those obtained by Venema et al. (1996). The LOQ values of the method were low enough to allow quantitative determination of salicylates content in 11 among 12 tested products. The amounts of *free SA* ranged from 3.34 to 7.69 $\mu g\ g^{-1}$ in spices, 0.31 to 4.37 $\mu g\ g^{-1}$ in lyophilized fruits and vegetables, and 0.02 to 0.68 $\mu g\ ml^{-1}$ in beverages, whereas *free + bound SA* content ranged from 7.72 to 35.61 $\mu g\ g^{-1}$ in spices, 1.18 to 34.04 $\mu g\ g^{-1}$ in lyophilized fruits and vegetables, and 0.05 to 0.70 $\mu g\ ml^{-1}$ in beverages (Table 2). The determined values of salicylate content in the tested food products are, where comparison is possible, in agreement with the data available in the literature (Venema et al. 1996, Wood et al. 2011). The remarkable differences in the *free SA* and *free + bound SA* content observed in the case of strawberry, cucumber, and oregano (approximately 50, 10, and 5-fold increase of SA content after acidic hydrolysis, respectively), together with obtained recovery rates for SA, confirm that applied initial sample extraction step allows efficient extraction of both *free SA* and its various derivatives. For comparison, Venema et al. (1996) reported about 20, 4, and 2-fold increase of SA content in these products after alkaline hydrolysis of the samples.

All these results, taken together, indicate that the presented method is characterized by an adequate accuracy and precision for fast and reliable determination of salicylates in spices, beverages, lyophilized fruits or vegetables, and presumably also in the other food items that can be freeze-dried. The use of the lyophilized products, which are much easier to handle than the fresh products, leads to a considerable unification of the assay and facilitates the sample preparation for HPLC analyses. Therefore, the developed method should be a versatile tool for routine assays.

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