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Simultaneous determination of salicylic, 3-methyl salicylic, 4-methyl salicylic, acetylsalicylic and benzoic acids in fruit, vegetables and derived beverages by SPME-LC-UV/DAD

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Graphical abstract

Highlights

- A new SPME-HPLC/UV(DAD) method is proposed.
- Salicylates and benzoic acid are determined simultaneously in fruits and vegetables.
- Overall, the proposed method is easy and cost-efficient.
- We believe that it could be potentially applied to other food commodities.

Abstract

Salicylic and benzoic acid are phenolic acids occurring in plant cells, thus they can be present in fruit and vegetables at various levels. They possess anti-inflammatory and antimicrobial properties, however they may induce symptoms and health problems in a small percentage of the population. Therefore, a low phenolic acid diet may be of clinical benefit to such individuals. In order to achieve this goal, the concentration of these substances in different food and beverages should be assessed. The present work describes for the first time a new method, based on solid phase microextraction (polydimethylsiloxane-divinylbenzene fiber) coupled to liquid chromatography with UV diode array detection, for the simultaneous determination of salicylic acid, 3-methyl salicylic acid, 4-methyl salicylic acid, acetylsalicylic acid and benzoic acid in selected fruit, vegetables and beverages.

All the aspects influencing fiber adsorption (time, temperature, pH, salt addition) and desorption (desorption and injection time, desorption solvent mixture composition) of the analytes have been investigated. An isocratic separation was performed using an acetonitrile-phosphate buffer (pH 2.8;

2 mM) mixture (70:30, v/v) as the mobile phase. The estimated LOD and LOQ values (µg/mL) were in the range 0.002-0.028 and 0.007-0.095. The within-day and day-to-day precision values (RSD %) were between 4.7 -6.1 and 6.6 - 9.4, respectively.

The method has been successfully applied to the analysis of fava beans, blueberries, kiwi, tangerines, lemons, oranges and fruit juice (lemon and blueberry) samples. The major advantage of the method is that it only requires simple homogenization and/or centrifugation and dilution steps prior to SPME and injection in the LC system.

Keywords: Salicylic acids; benzoic acid; SPME-HPLC; fruits; vegetables; beverages

"Dedicated to Professor Pier Giorgio Zambonin (Bari, Italy) on the occasion of his 80th birthday"

1. Introduction

Phenolic compounds are the largest and most widely distributed group of secondary metabolites in plants [1, 2]. Therefore, variable amounts of them can be found in fruits and vegetables even at high levels.They are used by plants for growth and development processes, as well as photosynthesis, transpiration, ion uptake, and transport [3-5]. In particular, salicylic acid (SA), its methyl- and acetyl-esters (denominated salicylates) and its glycoside derivatives are present at different concentrations in plant tissues [6, 7]: blackberries and blueberries; cantaloupes, dates, raisins, kiwis, guavas, apricots, green peppers, olives, tomatoes, radish and chicory mushrooms, some herbs and spices, legumes, seeds, nuts, cereals, almonds, water chestnuts [2, 8-12].

In the past few years, these natural compounds have received increasing interest concerning several controversial issues [13-15].The medicinal properties of salycilates have been known for a long time [7, 16-22]. Acetylsalicylic acid (ASA) is a well known drug (*aspirin*) used as an antipyretic, analgesic and antithrombotic agent [16, 17]. In addition, anticarcinogenic and antidiabetic effects have been recognized for ASA [18, 19]. It is a prodrug of SA which suppresses the activity of cyclooxygenase (COX) thus blocking prostaglandin synthesis [18, 20]. SA is also known to stimulate the adenosine monophosphate-activated protein kinase (AMPK) with consequent anticancer and antidiabetic effects [7, 21, 22]. Although a diet rich in salicylates might have positive effects on human health, there is a small percent of the population for which even a small dose of these compounds may be a problem. Some adults and children may develop symptoms and health problems from salicylates, which are dose-related. This is called 'Salicylate Sensitivity' or 'Salicylate Intolerance', which produces urticaria, angioedema, rhinitis, bronchial asthma and/or recurrent nasal polyps [23, 24].

The chronic nature of some of these clinical presentations may suggest an underlying etiology related to dietary salicylates. Thus, a low salicylate diet may be of clinical benefit to individuals affected by the above intolerance. This cannot be established, however, until the total salicylate content in food, that includes the contribution of SA and other salicylates, mainly methyl esters and glucosides of SA, is known. Thus, methods for their determinations in fruit, vegetables and related beverages are extremely useful.

Existing papers on this topic require complicated sample pre-treatment approaches and have been based on UV and fluorescence spectroscopy [25, 26], chromatographic techniques [2, 27-29], flow injection atomic absorption spectrometry [30] and electrochemistry [31, 32]. Furthermore, derivatives of salicylic acid have never been considered, thus making the assessment of the real dietary exposure to salycilates virtually impossible.

Very few data have also been reported to date [33, 34] on the simultaneous determination of SA and benzoic acids (BA), an active metabolite occurring in plants, which is a precursor of SA in the biosynthetic pathway [35]. It is widely used as a fungicidal and antimicrobial agent, and also in pharmaceutical preparations to help drug uptake and delivery after administration [36-39]. However, despite its widespread use, it could be very toxic since it can lead to benzene formation through a decarboxylation reaction [40-42]. Liquid chromatography with ultraviolet detection (LC-UV) methods have been reported [41, 43-45] for the determination of benzoic acid in fruits and derived products that required complex sample clean-up with organic solvents or solid-phase extraction The present work describes for the first time a new method. based on the solventless extraction technique known as solid phase microextraction (SPME) [46] coupled to LC-UV diode array (DAD), for the simultaneous determination of SA, some of its derivatives (3-methyl salicylic acid, 4-methyl salicylic acid, acetylsalicylic acid) and BA (see Figure 1) in fava beans, blueberries, kiwi, tangerines, lemons, oranges and fruit juice (lemon and blueberry) samples.

2. Material and methods

2.1 Chemicals

Salicylic acid, 3-methyl salicylic acid, 4-methyl salicylic acid, acetyl salicylic acid and benzoic acid were supplied by Sigma-Aldrich s.r.l. (MI, Italy).

Stock solutions (10 mg/mL) were prepared in ethanol and stored in the dark at +4 °C. Working solutions (concentration range 0.001-3 μg/mL) were prepared in water just before use in the presence of HCl (pH 2.0; 10 mM) with NaCl (0.3 g/mL).

All chemicals and organic solvents used (Sigma-Aldrich) were LC grade. The mobile phase was filtered through a 0.20 μm nylon membrane (Lab Service Analytica, Bologna, Italy).

2.2 Apparatus

The LC system (ThermoQuest, San Jose, CA) consisted of a Spectra System Pump, model P2000, an SPME interface (Supelco), with a standard six-port Rheodyne valve with a special fiber desorption chamber (total volume: 60μL) installed in place of the sample loop. The detector was a Spectra System model UV6000LP photodiode array (ThermoFinnigan,San Jose, CA) controlled by ChromQuest software running on a personal computer. The column used was a Phenomenex (Torrance, CA, USA) Kinetex C18, 4.6 mm x 100 mm (2.6 μm).

2.*3 Chromatographic and detection conditions*

The mobile phase was an acetonitrile-phosphate buffer (pH 2.8;2 mM) mixture (70:30, v/v). The flow rate was 0.9 mL/min and detection took place at room temperature. The detection wavelength was 336 nm (5 nm band-width). Spectra were acquired in the 220–380 nm range at the apex and on the ascending or descending part of each peak. Peak purity was checked by the technique of spectra overlaying, after normalization.

2.4 SPME conditions

Fibers coated with a 60 μm thick polydimethylsiloxane/divinylbenzene (PDMS/DVB, Supelco) film were used. A manual SPME device (Supelco) was used to hold the fiber. Extractions were performed in 15 mL amber vials (Supelco) with PTFE hole caps (Supelco) by direct immersion of the fiber in the sample solution under magnetic stirring. The extraction was carried out for 30 min at 20°C in HCl (pH 2.0; 10 mM), with 0.3 g/mLNaCl. Analyte desorption was performed in static desorption mode by soaking the fiber in the mobile phase into the desorption chamber of the interface for 5 min. Then the valve was changed to the inject position for 5 s. In order to ensure a complete desorption from the fiber, a blank SPME-chromatogram (dynamic desorption mode) was performed before the next extraction.

2.5 Samples collection and pre-treatment

All the samples, fresh or frozen, were purchased from local supermarkets. Fresh samples were processed within 24 h after removing the inedible parts. Fruits and legumes, fresh or frozen. Blueberries and fava beans, frozen by Orogel s.p.a., (Cesena, Italy). Kiwi, tangerines, lemons and oranges, were fresh and all of Italian origin. In addition, two different fruit beverages were selected, both made by Fruttagel s.p.a., (Ravenna, Italy). According to the labels, beverage 1 was a citrus juice (25%) and beverage 2 was a pure blueberry juice (100%).

Three sample pre-treatment approaches were optimized depending on the different samples.

A (fava beans, blueberries (thawed), peeled kiwi). Samples (10 g) were stirred for 30 min with 25 mL of 1% (w/v) Na_2CO_3; then homogenized and centrifuged for 5 min at 5000 rpm; the supernatant was transferred into a beaker and acidified to pH 2.0 with HCl (1M).

B (tangerines, lemons and oranges).Samples (100g) were homogenized and centrifuged for 5 min at 5000 rpm, obtaining 32.0±0.5, 40.2±0.3 and 56.3±0.3 mL of supernatants for tangerines, lemons and oranges, respectively. Then 15.0 mL of volumes of the supernatant solution were transferred into a beaker and acidified to pH 2 with HCl (1M).

C (fruit juices). Sample were diluted (1:10) with HCl (pH 2.0; 10 mM) and centrifuged for 5 min at 5000 rpm.

Finally, all supernatants were filtered (0.8 μm Minisart filters,Whatman Limited, Maidstone, UK) and subjected to SPME-LC analysis. In the case of procedures A and B, it was necessary to further dilute the samples (1:10) with 10 mM HCl before analysis.

Quantitation was performed using the standard addition method. Samples were spiked 2 h before being processed. Samples were always analyzed in triplicate. Analyte concentrations were estimated from absolute values of the x-intercepts of the (unweighted) regression lines obtained.

3. Results and discussion

3.1 SPME-LC optimization. Firstly, all the parameters relevant to the resolution of the analytes under study, i.e. stationary phase, mobile phase composition and flow rate, were carefully considered and optimized. The ideal conditions are reported in the experimental section.

Then, all the factors affecting adsorption of the analytes on the SPME fiber (time, temperature, pH, ionic strength) and their desorption in the SPME–LC interface (desorption mode and desorption mixture composition) were investigated. Figure 2a reports the SPME extraction time profiles obtained by plotting the area counts versus the extraction time, obtained at 20°C. As apparent, after 60 minutes the equilibrium was reached for all the target analytes. In any case, since it is also possible to obtain good extraction yields and reliable analysis in non-equilibrium conditions, an extraction time of 30 minutes was chosen for further experiments. A response decrease was observed at 50°C (data not shown).

A significant decrease in the extraction efficiency was observed by varying the pH in the range 2.0–5.0, due to a strong dependence of the SPME extraction yield of acidic compounds on the pH value [47].Therefore further experiments were performed at pH 2.0.

Salt addition usually improves the recovery percentage, especially in the case of polar compounds that are difficult to extract. Thus, experiments were performed by increasing the ionic strength of the

extraction solutions (sodium chloride addition from 0 to 0.3 g/mL), which produced a progressive signal enhancement for all the analytes. The results obtained are shown in Figure 2b.

As far as the analyte desorption in the SPME-LC interface is concerned, the fiber was soaked in the mobile phase for different periods of time (static desorption mode) before injection. The best conditions (recovery of 78.0 ± 1.3%) were achieved after 5 min of static desorption time followed by a valve switch to load position 20 s after injection, in order to preserve satisfactory chromatographic efficiency.

The SPME enrichment factors for each analyte were also calculated. Figure 3 shows a comparison of the LC–UV/DAD chromatograms obtained (a) by directly injecting (60 μL) a standard solution of the analytes at the concentration level of 0.25 μg/mL and (b) by subjecting the same solution to SPME. The enrichment factors for each component were calculated as the ratio between the signals in trace (b) and (a). The obtained values were in the range 17.42 ± 0.75 (3-MeS) – 0.74 ± 0.09 (AAS).

3.2 Linear range, detection limits and precision.

The calibration curves were linear from the LOQ values of each analyte over at least two orders of magnitude of concentration with correlation coefficients better than 0.998 and an intercept not significantly different from zero at 95% confidence level.

The estimated LOD and LOQ, calculated as three- and ten-fold the standard deviation of the intercept of the calibration curve, are shown in Table1.

The within-day precision of the method was investigated on standard solutions at the concentration level of 0.05 μg/mL for SA, 3-MeSA, 4-MeSA and BA, and 0.2 μg/mL for ASA, by performing three replicates daily. The same solutions were analyzed three times each day, for a period of five days, for the day-to-day precision evaluation. The relevant data are also reported in Table 1.

3.3 Application to fruits, legumes and beverages

The developed procedure was then applied to the analysis of fruit, legume and beverage samples subjected to the sample pre-treatment reported in the Experimental Section. Figure 5 shows, as an example, the SPME-LC-UV/DAD chromatograms relevant to the analysis of some selected samples, while Table 2 summarizes all the results obtained in the present study. As is apparent from Table 2, the target analytes were found in different real matrices and are well separated by matrix interferents, clearly showing the applicability of the method. SA and BA were detected in all the analyzed samples, with the only exception of oranges (trace Bc); on the contrary, ASA was only found in orange samples. SA, 3-MeSA and 4-MeSA were detected in fava beans (trace Ab), blueberries (traces Ac and Ca) and lemons (trace Bb). The presence of SA and BA in some of the selected samples was expected, since it is well known that these metabolites can be present at significant levels (>0.01

mg/g) in selected fruits, vegetables and fruit juices [2, 27-29]. The estimated concentrations of SA were in agreement with previously reported data [2, 27-29]. The present method also permitted the determination for the first time of 3-MeSA and 4-MeSA in blueberries, fava beans and lemons.

4.Conclusions

The simultaneous determination of SA, some of its derivatives (3-methyl salicylic acid, 4-methyl salicylic acid, acetylsalicylic acid) and BA by SPME- LC-UV/DAD was accomplished for the first time. The LC separation of the target compounds was achieved working in isocratic conditions.

All SPME parameters, (i.e. extraction time, temperature, pH, salt addition, desorption and injection time, desorption solvent mixture composition) have been carefully studied.

The potential of the described procedure is demonstrated by its ability to detect the analytes in several real matrices such as fava beans, blueberries, kiwi, tangerines, lemons oranges and fruit juice (lemon and blueberry) samples. Real sample analysis required only simple homogenization and/or centrifugation and dilution steps prior to SPME and injection in the LC system, and the addition of a minute amount of organic solvent. Work is underway in our laboratory on how the method could be applied to the determination of the target compounds in other food commodities.

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Table 1. Estimated LOD and LOQ for the target compounds and precision of the developed SPME-LC-UV/DAD method.

Analyte	LOD (μg/mL)	LOQ (μg/mL)	Within-day (RSD %)	Day-to-day (RSD %)
SA	0.002	0.007	5.6	7.8
3-MeSA	0.003	0.012	4.7	6.7
4-MeSA	0.003	0.011	5.0	7.5
ASA	0.028	0.095	6.1	9.4
BA	0.003	0.010	5.4	6.6

Table 2. Estimated concentrations of the target compounds found in selected food samples using SPME-LC-UV/DAD

Food	SA (μg/g)	3-MeSA (μg/g)	4-MeSA (μg/g)	ASA (μg/g)	BA (μg/g)	Total (μg/g)
Kiwi	1.00± 0.04				1.15± 0.06	2.15
Beans	1.39± 0.07	4.37± 0.13	0.92± 0.06		4.13± 0.09	10.80
Blueberries	1.33 ± 0.06	0.8 ± 0.07	24.00 ± 0.16		1.98 ±0.08	28.10
Tangerines*	0.06± 0.05				0.08±0.09	0.14
Lemons *	0.26±0.01		0.34±0.04		0.35±0.01	0.95
Oranges*				1.01±0.04		1.01
Beverage 1	1.20± 0.05				0.46± 0.05	1.66
Beverage2	0.30 ± 0.01	0.30 ± 0.01	0.20 ± 0.01		0.58 ± 0.03	1.38

* edible fruit/ fruit juice (g/mL): 3.30 g/mL Tangerines; 2.40 g/mL Lemons; 1.80 g/mL Oranges

Figure Captions

Fig. 1.Chemical structures of salicylic acid (a), 3-methyl salicylic acid (b), 4-methyl salicylic acid (c), acetylsalicylic acid (d) and benzoic acid (e).

Fig. 2.a) Extraction time profiles obtained for the target compounds (0.25 μg/mL) at 20°C; SA (□); 3-MeSA (♦); 4-MeSA (x); ASA (■); BA (▲); b) Effects of NaCl additions on the SPME extraction of the analytes.

Fig. 3. a) LC–UV/DAD chromatogram relevant to the direct injection of a standard mixture at the concentration level of 0.25 μg/mL (60 μL injected); b) SPME-LC–UV/DAD chromatogram of the same solution.

Fig. 4. SPME-LC–UV/DAD chromatograms relevant to: A) solid fruit: kiwi (a); fava beans (b); blueberries (c); B) citrus juice : tangerine (a); lemons (b); oranges (c); C) commercial fruit juices: beverage 1 (a); beverage 2 (b)

Figure 1

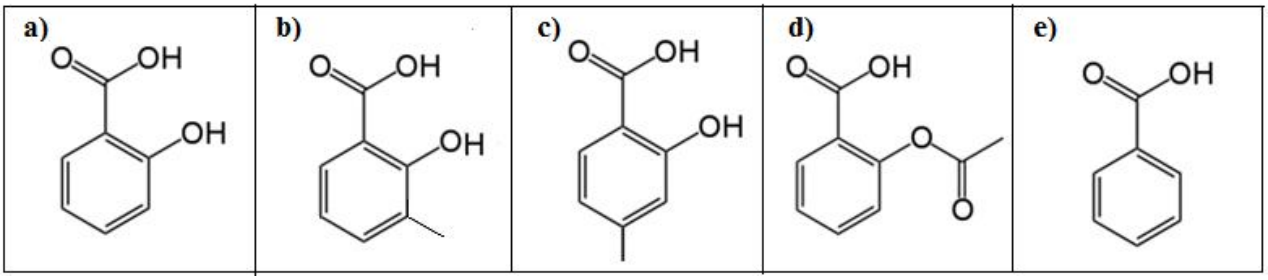

Figure 2

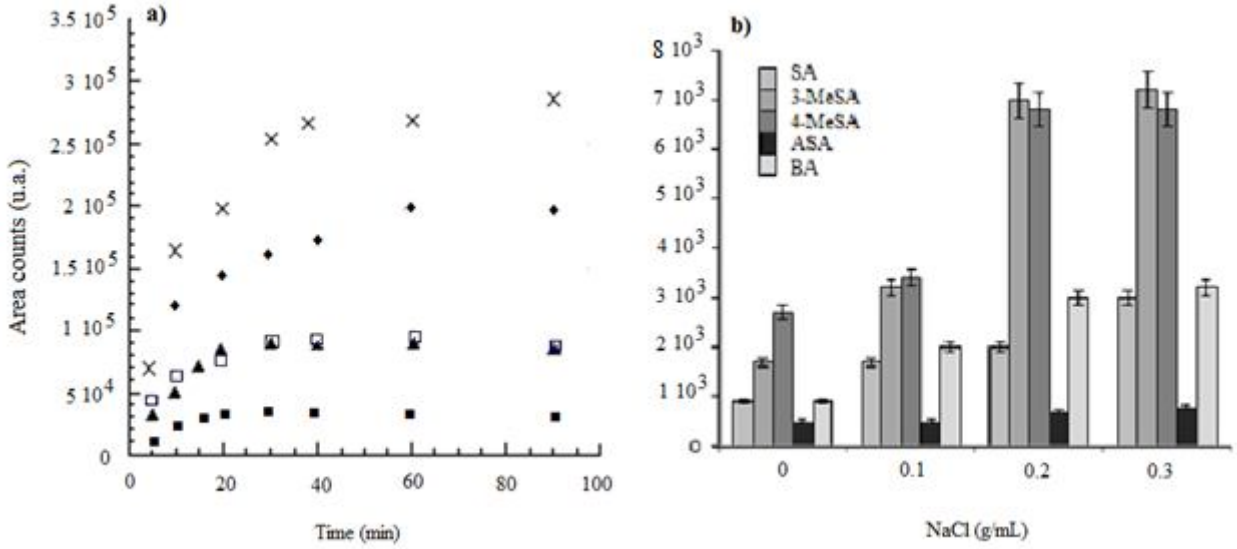

Figure 3

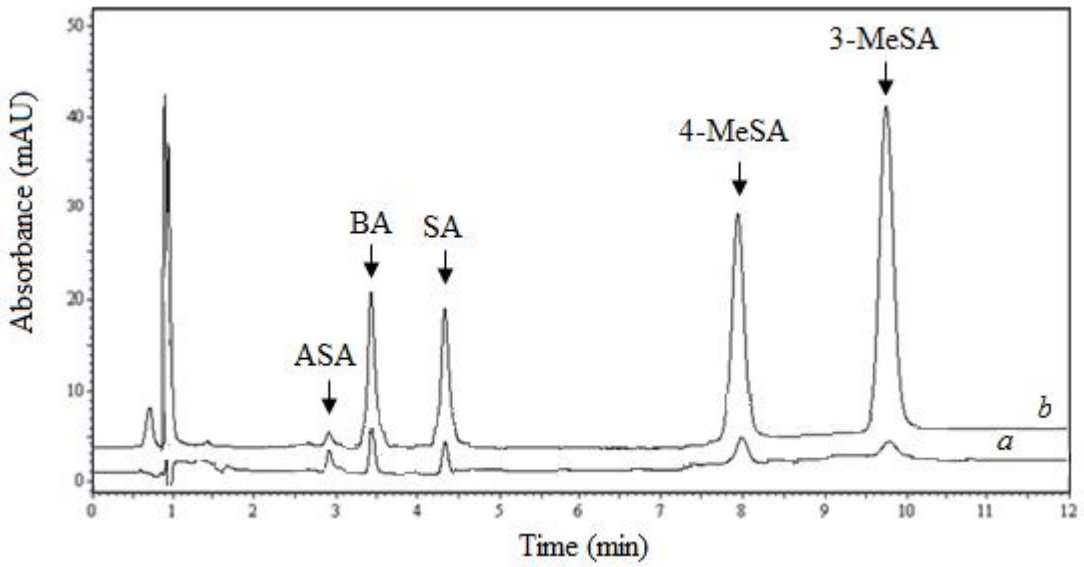

Figure 4