Short communication

A salting out system for improving the efficiency of the headspace solid-phase microextraction of short and medium chain free fatty acids

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A R T I C L E   I N F O

A B S T R A C T

Given the importance of short and medium chain free fatty acids (FFAs) in several fields, this study sought to improve the extraction efficiency of the solid-phase microextraction (SPME) of FFAs by evaluating salting out agents that appear promising for this application. The salts ammonium sulfate ($(NH_4)_2SO_4$) and sodium dihydrogen phosphate (NaH_2PO_4) were tried on their own and in combination (3.7/1), in four different total amounts, as salting out agents in the headspace–SPME–gas chromatographic (HS–SPME–GC) analysis of the FFAs from acetic acid (C2) to decanoic acid (C10). Their performance in a model system of an aqueous standard mixture of FFAs at a pH of 3.5 was compared to that of the more commonly used sodium chloride (NaCl) and sodium sulfate (Na_2SO_4). All of the salts and salt systems evaluated, in proper amount, gave improved results compared to NaCl (saturated), which instead gave interesting results only for the least volatile FFAs C8 and C10. For C2–C6, the salt system that gave the best results compared to NaCl was $(NH_4)_2SO_4/NaH_2PO_4$, in the highest of the four amounts evaluated, with factor increases between 1.2 and 4.1-fold, and NaH_2PO_4, between 1.0 and 4.3-fold. The SPME extraction efficiency given by the mixture $(NH_4)_2SO_4/NaH_2PO_4$ was also assessed on biological and food samples, confirming that overall it performed better than NaCl.

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1. Introduction

The amount and composition of free fatty acids (FFAs) in food as well as in other biological samples are important from several points of view. In food products such as cheese, FFAs afford the characteristic flavor and are typically related to the production process and product quality [1–3]. In contrast, FFAs in butter, milk or cream are undesirable because they cause a cheese-like off-flavor [4], while acetic acid above a certain level in wine fosters the growth of acetic bacteria and causes spoilage. Similarly, medium chain free fatty acids (MCFFAs) (C6, C8, C10) are also found in wine and other food products, and their amount is also important, since it can impart an off-flavor when it exceeds certain values [5]. Short chain free fatty acids (SCFFAs), namely acetic (C2), propionic (C3) and butyric acid (C4), are also important from a physiological point of view, since they are fermentation products of dietary fiber and are

found in the intestinal content in amounts depending on diet, gut microflora, and state of health [6]. Thus the analysis of SCFFAs and MCFFAs plays an important role in evaluating the quality of a food, and also in establishing relationships between nutrition and health in humans and animals. The analysis of this kind of analytes can be conveniently performed by solid phase microextraction (SPME) and gas chromatography, whose applications have been reported in several studies [1,4,7–13]. In the headspace (HS)–SPME of aqueous or suspended/solubilized samples, the efficiency of the extraction is generally improved by using a salting out agent able to increase the concentration of the apolar analytes in the HS, and thus in the SPME fiber coating, because this increases the ionic strength of the solution and leads to decreased solubility of these compounds in the solution. The use of bivalent salts would further improve extraction because they increase ionic strength more than monovalent ions do. Nevertheless a relatively low number of studies exploits bivalent or multivalent salts [1,7,14,15].

Thus, in the present study, given the importance of FFAs analysis in several fields, it was aimed at improving the HS–SPME extraction efficiency of SCFFAs and MCFFAs by using a salt mixture never

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before used to this purpose: $(NH_4)_2SO_4/NaH_2PO_4$. The effect of the salt pair $(NH_4)_2SO_4/NaH_2PO_4$ was studied and compared to the individual salts $(NH_4)_2SO_4$ and NaH_2PO_4 as possible salting out agents, to evaluate whether they would perform better than the most commonly used salts NaCl or Na_2SO_4.

2. Materials and methods

2.1. Standards, reagents and solvents

The FFAs standards C2, C3, C4, C5, iC5, C6, iC6, C8, C10, anhydrous Na_2SO_4, NaH_2PO_4, $(NH_4)_2SO_4$ and sulfuric acid were purchased from Sigma–Aldrich (Milan, Italy). Water used in this study was deionized (>8 MΩ cm resistivity) using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA). NaCl was purchased from Carlo Erba.

2.2. HS–SPME

2.2.1. Evaluation of $(NH_4)_2SO_4/NaH_2PO_4$ amount and comparison of different salting out agents: $(NH_4)_2SO_4/NaH_2PO_4$, $(NH_4)_2SO_4$, NaH_2PO_4, Na_2SO_4, NaCl

The extraction of the volatile compounds was performed by SPME using the divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm coated fiber (DVB/Car/PDMS fiber) purchased from Supelco (Bellefonte, PA, USA). Before each analysis, the fiber was conditioned in the GC injector at 260 °C for 15 min. The salt effect was evaluated by analyzing 3 mL of an aqueous standard mixture of FFAs (C2, C3, C4, C5, iC5, C6, iC6, C8, C10) at the concentrations of 300, 60, 10, 1, 1, 0.2, 0.2, 0.2, 0.2 mg L^{-1}, respectively, in a 12 mL screw cap vial (Agilent Technologies, Santa Clara, CA, USA) keeping the mixture under magnetic stirring (500 rpm) using a small magnet (1.5 cm length, 4 mm diameter). The following amounts of salts were used: 4 g of NaCl (saturated solution) or different total amounts of the salt mixture $(NH_4)_2SO_4/NaH_2PO_4$, in a ratio of 3.7/1, namely: 0.84 g ($(NH_4)_2SO_4/NaH_2PO_4$-1), 1.68 g ($(NH_4)_2SO_4/NaH_2PO_4$-2), 2.52 g ($(NH_4)_2SO_4/NaH_2PO_4$-3) or 3.36 g ($(NH_4)_2SO_4/NaH_2PO_4$-4) or amounts of Na_2SO_4, $(NH_4)_2SO_4$, or NaH_2PO_4 such as to saturate each solution and to obtain final equal volumes. The pH of the solutions of NaCl, $(NH_4)_2SO_4$ and of Na_2SO_4 was adjusted to 3.5 with sulfuric acid (10 μL at 0.06 mol L^{-1} for NaCl and Na_2SO_4; 20 μL at 0.6 mol L^{-1} for $(NH_4)_2SO_4$ while NaH_2PO_4 and $(NH_4)_2SO_4/NaH_2PO_4$ solutions spontaneously reached a pH of 3.5 due to the hydrolysis of the salts. The solutions prepared were extracted at 35 °C, incubating for 30 min and extracting for 60 min.

2.2.2. Analyses of cheese samples

The analyses were conducted on four different samples of grated cheeses purchased from a supermarket. Each sample (1.5 g) was weighed in a 15 mL screw cap vial (Supelco, Bellefonte, PA, USA), into which a small magnet and 4 g of NaCl or 3.36 g of the salt mixture $(NH_4)_2SO_4/NaH_2PO_4$ were added. Then, 3 mL of internal standard aqueous solution (iC6, 3.333 mg L^{-1}) and sulfuric acid were added to obtain a pH of 3.5 in each sample. The samples were extracted at 35 °C for 15 min after incubation under magnetic stirring for 15 min.

2.2.3. Analyses of wine samples

Analyses were conducted on four different samples of wines purchased from a supermarket: two red wines (wine 1 and wine 2) and two white wines (wine 3 and wine 4). The sample (3 mL) was put into a 12 mL screw cap vial, to which were added a small magnet, 4 g of NaCl or 3.36 g of the salt mixture $(NH_4)_2SO_4/NaH_2PO_4$ and 4 μL of iC6 aqueous solution (1000 mg L^{-1}). The samples were extracted at 35 °C for 30 min after incubation under magnetic stirring for 15 min. In the SPME–GC–MS analysis a solvent delay of 3.0 min was set so that the high ethanol amount would not overload the filament work.

2.2.4. Analyses of rat feces samples

Four different samples of rat feces (from four different rats) provided by a biology laboratory were analyzed. The sample (1 g) was homogenized under magnetic stirring with deionized water (5 mL). Of this mixture, 0.15 g were weighed in a 12 mL screw cap vial, to which were added a small magnet and 4 g of NaCl or 3.36 g of $(NH_4)_2SO_4/NaH_2PO_4$, 3 mL of internal standard aqueous solution (iC6, 0.233 mg L^{-1}) and sulfuric acid to obtain a pH of 3.5 in each sample. The SPME extractions were carried out with incubation of 15 min and extraction time of 30 min.

2.3. GC–MS/FID analyses

The gas chromatographic analyses were accomplished using an Agilent Technologies 6850 GC (Santa Clara, CA, USA) equipped with a split/splitless injector and a flame ionization detector (FID). A GC 6850 equipped with a split/splitless injector and a single quadrupole mass spectrometer detector 5973 (both from Agilent Technologies) was also used to confirm the identity of the analytes in the real samples analyzed. The capillary chromatographic column used was a nitroterephthalic acid modified polyethylene glycol (PEG) column (DB-FFAP, 25 m, 0.32 mm i.d., 0.25 μm film thickness, purchased from Agilent Technologies). The GC analysis was performed by desorbing volatiles extracted in the GC injector maintained at 260 °C. The injection was performed in splitless mode (splitless time 4 min), and the insert used was a 0.75 mm i.d. liner for SPME (Supelco, Bellefonte, PA, USA). The oven temperature was initially set at 35 °C for 5 min, programmed at a rate of 10 °C min^{-1} to 220 °C, where it was held for 1.75 min, resulting in a total run time of 15 min. The carrier gas was hydrogen for GC-FID, at a flow rate of 3.0 mL min^{-1} and helium for GC–MS at a flow rate of 1.5 mL min^{-1}. The flame ionization detector temperature was maintained at 230 °C. MS operational conditions were: electron ionization (EI) at 70 eV; transfer line and ion source temperature: 250 °C; quadrupole temperature: 150 °C; and mass range: m/z 29–300. The identity of FFAs detected in the real samples was confirmed by comparison of their retention times and their mass spectra with those of authentic standards and with reference spectra from the US National Institute of Standards and Technology (NIST, 2008).

2.4. Statistical analysis

Data were submitted to one-way analysis of variance (ANOVA) in order to assess significant differences ($P < 0.05$) between the extraction efficiencies obtained by the different systems evaluated. The software used was PAST [16]. Experiments carried out with the aqueous standard mixture of FFAs were performed in triplicate analyses while experiments with real samples were performed with five replicate analyses.

3. Results and discussion

3.1. Evaluation of the salting out effect given by the salt combination $(NH_4)_2SO_4/NaH_2PO_4$ and comparison with $(NH_4)_2SO_4$, NaH_2PO_4, Na_2SO_4 and NaCl

The main aim of this study was to evaluate the salting out effect given by promising salting out agents, namely the mixture $(NH_4)_2SO_4/NaH_2PO_4$, in the SPME extraction of SCFFAs and MCFFAs, and to compare them with more commonly used salting out agents. The idea arose from a study [17] where the salt combination $(NH_4)_2SO_4/NaH_2PO_4$ used in a ratio of 3.7/1, gave interesting results