Selective removal of phosphate for analysis of organic acids in complex samples

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ABSTRACT

Accurate quantitation of compounds in samples of biological origin is often hampered by matrix interferences one of which occurs in GC-MS analysis from the presence of highly abundant phosphate. Consequently, high concentrations of phosphate need to be removed before sample analysis. Within this context, we screened 17 anion exchange solid-phase extraction (SPE) materials for selective phosphate removal using different protocols to meet the challenge of simultaneous recovery of six common organic acids in aqueous samples prior to derivatization for GC-MS analysis. Up to 75% recovery was achieved for the most organic acids, only the low pKa tartaric and citric acids were badly recovered. Compared to the traditional approach of phosphate removal by precipitation, SPE had a broader compatibility with common detection methods and performed more selectively among the organic acids under investigation. Based on the results of this study, it is recommended that phosphate removal strategies during the analysis of biologically relevant small molecular weight organic acids consider the respective pKa of the anticipated analytes and the detection method of choice.

1. Introduction

Carbohydrates and their metabolites, i.e. organic acids and sugar alcohols, have always been of major interest to biological research, mostly due to their nutritional value [1,2] and their biological function in health and disease [3–5]. Thus, the profiling of carbohydrates by gas chromatography–mass spectrometry (GC-MS) as established decades ago [6] has meanwhile been extended to simultaneous analysis of carbohydrates, organic acids and amino acids, and finally became a standard technique in the field of metabolomics [7–9].

Usually, samples of biological origin are very complex mixtures in terms of the chemical nature of their components and their respective concentration in the sample. Often, a few abundant compounds dominate the composition of the sample while many others are present at low concentration hampering their quantitative analysis [10,11]. Within this context, matrix interference has always been a concern, particularly at the lower limits of quantitation [11–13]. Consequently, a general strategy for correct quantitation of less abundant target compounds is the selective removal of the highly abundant compounds from the sample to avoid matrix

effects when increasing the sample amount for analysis [14]. A prominent example of matrix interference is the ubiquitous presence of phosphate in many biological applications [15]. Phosphate is an important constituent of many biochemical pathways (e.g. in the form of phospholipids, -proteins or nucleotides) and a core constituent of the intracellular buffer. Furthermore, many buffers used for *in vitro* investigations of biomolecules also contain phosphate which frequently makes it the most abundant compound in such preparations [16]. Selective removal of excess phosphate is indispensible for the analysis of less abundant compounds with GC-MS to avoid overload of the analytical process and the problems associated with it.

The separation of phosphate as a polar, ionic compound from neutral target analytes can be achieved by reversed-phase solid phase extraction (SPE). In contrast, a separation targeting particularly the polar, small molecular weight organic acids has not been accomplished yet [17,18]; available protocols remove all acids with low pKa [11]. However, many small-molecular weight organic acids are of particular interest to biological investigations, such as the constituents of the citric acid cycle or the sugar acids. Phosphate is also an acidic compound of similar molecular weight so that achieving a separation from these acids is indeed challenging [19]. Thus, liquid partitioning applied for phosphate removal was found to badly recover polar organic acids [11]. The commonly used phosphate precipitation is not only a fairly complex and lengthy process

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[20] but was also found to result in lower recovery after solid phase extraction of these analytes as well and optimization of the salt concentration for precipitation was recommended [11]. Recovery loss of many substances was observed as well when Carrez precipitation was used for sample preparation [15] and for carbohydrates after precipitation of barium sulfate [21]; for these consequences of precipitation in general, co-precipitation of the target analytes was discussed. Thus, in this work we explored the potential of SPE for developing an easy, high-recovery phosphate removal procedure without additional steps such as liquid/liquid extraction [18].

The utilization of anion exchange sorbent materials deemed particularly promising to selectively retain phosphate based on differential deprotonation of the acids by varying the pH and polarity of the sample solvent and eluent. This study reports on a screening of 17 silica- and polymer-based anion exchange solid phase materials of common manufacturers to particularly recover, for the first time, common small molecular weight organic acids in addition to carbohydrates from an aqueous phosphate buffer. We present a comparison of the obtained recoveries and discuss the impact of different material characteristics on selective retention. The achievements are compared to the approaches of phosphate precipitation and selective derivatization prior to GC-MS analysis.

2. Materials and methods

2.1. Chemicals and SPE cartridges

Sodium lactate (LA), threonic acid hemicalcium salt (ThoA), glyceric acid hemicalcium salt (GroA), gluconic acid sodium salt (GlcA), gluconic acid delta lactone (GDL), erythritol (Ery-ol), ribitol (Rib-ol) and methoxyamine hydrochloride (MOA) were obtained from Sigma–Aldrich, Taufkirchen, Germany; citric acid (CA), glucose (Glc), fructose (Fru), mannitol (Man-ol), ribose (Rib), tartaric acid (TA), sodium fluoride (NaF), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4), calcium dichloride hexahydrate ($CaCl_2 \cdot 6\,H_2O$), formic acid (FA) and methanol (MeOH) from Merck KGaA, Darmstadt, Germany. N-Methyl-N-trifluoroacetamide (MSTFA) was from Macherey-Nagel, Düren, Germany, and pyridine (PY) from Sigma-Aldrich, Steinheim, Switzerland.

Samples of 17 anion exchange (AEX) solid-phase extraction (SPE) columns were kindly provided as follows: (i) silica-based SPE columns: Strata SAX (Phenomenex, Aschaffenburg, Germany), Hypersil SAX (Thermo Scientific, Dreieich, Germany), Isolute PE-AX and SAX (Biotage, Uppsala, Sweden), Chromabond DMA and SB (Macherey-Nagel, Düren, Germany), SepPak QMA (Waters, Manchester, UK), SiliaPrep C8/SAX (SiliCycle, Quebec, Canada); (ii) polymer-based SPE columns: Strata X-AW and X-A (Phenomenex, Aschaffenburg, Germany), Chromabond HR-XA and PS-OH (Macherey-Nagel, Düren, Germany), HyperSep Retain-AX (Thermo Scientific, Dreieich, Germany), Oasis MAX (Waters, Manchester, UK), HybridSPE-Phospholipid and Supelclean ENVI-Carb Plus (Sigma-Aldrich, Taufkirchen, Germany), Bond Elut Plexa PAX (Agilent, Waldbronn, Germany).

2.2. SPE procedure and sample analysis

Samples were prepared containing 1.5 mM phosphate and 50 μM of the 13 analytes (structures are shown in Table S1, supplement), namely lactic (LA), glyceric (GroA), gluconic (GlcA), threonic (ThoA), tartaric (TA), and citric (CA) acid, gluconic acid delta lactone (GDL), ribose (Rib), glucose (Glc), fructose (Fru), erythritol (Ery-ol), ribitol (Rib-ol), and mannitol (Man-ol) dissolved in the respective eluent (see below). All SPE cartridges were conditioned with 6 M NaCl solution, followed by 5 mL water and 5 mL methanol. 85 μL

of the sample was applied to the SPE columns containing 30 mg adsorbent and eluted using a vacuum manifold following five different schemes employing 1.2 mL of either (i) formic acid at pH 2.4 (75 mM FA) or (ii) formic acid at pH 3.6 (0.53 mM FA) followed by a second step with 1.2 mL (i) 100 mM NaF at pH 2.4 or (ii) 100 mM NaF at pH 3.6, respectively, or, alternatively, (iii) 100 mM NaF/75 mM FA, (vi) 100 mM NaF/0.53 mM FA, and (v) 2% FA (v/v, 530 mM FA) in methanol as recommended for the polymeric materials (Table 1). Eluates were collected and dried (3–5 h depending on eluent composition) before derivatization using a vacuum centrifuge (Eppendorf, Hamburg, Germany).

For derivatization in presence of metal cations, 1.5 mM or, alternatively, 0.75 mM calcium dichloride was added to the sample; the supernatant was transferred to a fresh tube and evaporated to dryness before derivatization.

The general derivatization procedure was adopted from [15,22] incubating the sample in 30 μL of 20 mg/mL MOA in pyridine for 90 min at 30 °C and further 30 min at 37 °C after adding 55 μL MSTFA.

The sample mix with or, alternatively, without 1.5 mM phosphate was also (i) sonicated (soni) instead of shaken, and (ii) incubated without MOA, and (iii) incubated by shaking 30 min at 37 °C in 85 μL MSTFA only (without MOA/pyridine).

All experiments were carried out at least in triplicates. Samples were analyzed by GC-MS on a Trace GC Ultra coupled to a MAT95 XP sector field mass spectrometer (Thermo Electron, Bremen, Germany) as described earlier [15]. Identification of the analytes was confirmed by separate analyses of the reference compounds and library search with NIST 05 (National Institute of Standards and Technology, Gaithersburg, MD, USA).

Response of the baseline-separated target analytes was assessed as peak areas of the respective selective mass traces listed in Table S1 (supplement) and relative recoveries are expressed as the response ratios to the sample not subjected to any sample preparation other than evaporation and derivatization (recovery standards). 85 μL of the dried compound mix without phosphate was prepared as a recovery standard for the target compounds; 85 μL of a 100 μM phosphate solution was dried as a recovery standard for phosphate. For the relative signal response of 1.5 mM phosphate after SPE, the response area of phosphate in the sample was divided by 15 times the corresponding response of the 100 μM phosphate standard sample because response of 1.5 mM phosphate entered saturation outside the linear range and produced memory effects.

3. Results and discussion

3.1. Anion exchange SPE – general considerations for all adsorbents

We screened the performance of 17 silica- and polymer-based anion exchange materials from different manufacturers for selective removal of phosphate from aqueous solution. The phosphate concentration of 1.5 mM in our samples was selected as the maximum concentration of phosphate not exceeding an expected overall ionic strength of 2 mM (elution at pH 2.4) and 5 mM (elution at pH 3.6), respectively, for the whole sample solution containing phosphate, formate, and the target acids. The set of analytes was selected to be a mix of biologically important representatives of carbohydrates and related compounds with particularly low pKa. An additional criterion was the proper separation in subsequent GC-MS analysis. Table S1 (supplement) gives an overview of the structure and pKa values of the target compounds, dihydrogen phosphate and formic acid (the latter was used for pH adjustment).