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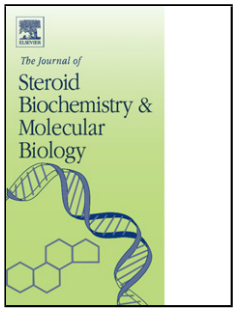

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Fast liquid chromatography-mass spectrometry reveals side chain oxysterol heterogeneity in breast cancer tumour samples

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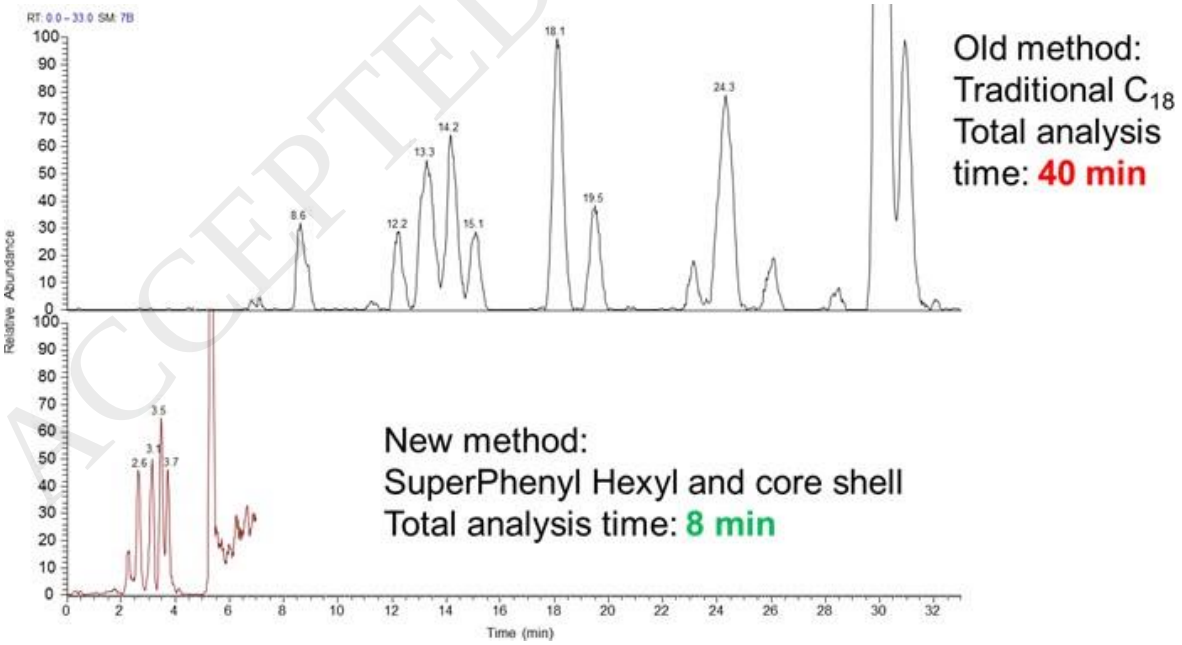
Declaration of interest: none

Graphical abstract



Highlights

- Fast LC-MS method for sensitive side chain oxysterol determination in breast cancer tumour samples
- Multiple slices from same tumours reveals oxysterol heterogeneity
- No significant differences in oxysterol content between ER-positive and ER-negative breast cancer
- Correlation between esterified and free 27-hydroxycholesterol in breast cancer tumours

Abstract

Oxysterols can contribute to proliferation of breast cancer through activation of the Estrogen Receptors, and to metastasis through activation of the Liver X Receptors. Endogenous levels of both esterified and free sidechain-hydroxylated oxysterols were examined in breast cancer tumours from Estrogen Receptor positive and negative breast tumours, using a novel fast liquid chromatography tandem mass spectrometry method. Multiple aliquots of five milligram samples of 22 tumours were analysed for oxysterol content to assess intra- and inter-tumour variation. Derivatization was performed with Girard T reagent (with and without alkaline hydrolysis) and sample clean-up was performed using a robust automatic on-line column switching system ("AFFL"). Oxysterols were separated isocratically on a 2.1 mm inner diameter column packed with ACE SuperPhenylHexyl core shell particles using a mobile phase consisting of 0.1 % formic acid in H_2O/methanol/acetonitrile (57/10/33, v/v/v) followed by a wash out step (0.1 % formic acid in methanol/acetonitrile, 50/50, v/v). The total analysis time, including sample clean-up and column reconditioning, was 8 minutes (80 % time reduction compared to other on-line systems). Analysis revealed large intra-tumour variations of sidechain oxysterols, resulting in no significant differences in endogenous oxysterols levels between Estrogen Receptor positive and Estrogen Receptor negative breast cancers. However, a correlation between esterified and free 27-hydroxycholesterol was observed. The same correlation was not observed for 24S-hydroxycholesterol or 25-hydroxycholesterol. The oxysterol heterogeneity of tumour tissue is a critical factor when assessing the role of these lipids in cancer.

Abbreviations

AFFL – Automatic filtration and filter back-flush

BCa – Breast cancer

ScOHC – Sidechain hydroxycholesterol

Keywords

- Oxysterols
- Breast cancer
- On-line sample clean-up
- 27-hydroxycholesterol
- Liquid chromatography-mass spectrometry

1. Introduction

1.1 Oxysterols and breast cancer

Breast cancer (BCa) is the most commonly diagnosed cancer among woman [1] and elevated LDL-cholesterol is a predictor of poor outcomes [2]. Oxysterols are cholesterol metabolites and the different isomers possess diverse biological roles in development and numerous diseases [3-8]. Oxysterols can be formed by either enzymatic or non-enzymatic oxidation of cholesterol. In breast cancer, the enzymatically formed 27-hydroxycholesterol (27-OHC) is of particular interest; In BCa, 27-OHC induces cell proliferation and tumour growth via its ability to bind and activate the Estrogen Receptor (ER) [9, 10] thus being at least part of the molecular evidence that links elevated cholesterol with BCa [9]. 27-OHC also activates the liver X receptor (LXR) leading to enhanced expression of transcription factors that promote the epithelial to mesenchymal transition [9, 11], and modifies immune γδ-T cells and polymorphonuclear-neutrophil function to promote ER-negative BCa metastasis [12]. A related sidechain oxysterol, 25-OHC, has also been found elevated in the circulation of BCa patients under going treatment for metastatic disease [13]. In BCa research, the focus has therefore been mainly on the 27-hydroxycholesterol [10, 14, 15]. However, the other enzymatically formed sidechain oxysterols (scOHC) can play roles, as e.g. LXR activators. In addition, little is known regarding the levels of oxysterols in ER-positive and ER-negative BCa tissue. Oxysterols can, as cholesterol, exist either as free sterols or bound to fatty acids (esterified) or sulfonated species [16]. In BCa neither the relative abundance, nor the roles of these modifications are well understood. Hence, methods for studying oxysterols in breast cancer tissues are needed.

1.2 Measurement of oxysterols

Traditionally, gas chromatography mass spectrometry (GC-MS) has been used for determination of sterols, such as oxysterols, after derivatization with trimethylsilyl (TMS) to increase volatility [17-19]. This procedure usually includes an alkaline hydrolysis step, resulting in the determination of total oxysterol, including both free and esterified oxysterols, as well as sulfonated oxysterols. Liquid chromatography mass spectrometry (LC-MS) is increasingly used for oxysterol determination [18]. The neutral nature of the sterol structure makes ionization of oxysterols using electrospray ionization (ESI) difficult, and derivatization is performed to "charge-tag" the analytes (e.g. with picolinyl, N,N-dimethylglycine (DMG) or Girard P and T [18, 20-22]. With charge-tagging, LC-MS surpasses GC-MS in sensitivity [23]. In our hands, Girard derivatization has both been highly robust and allowed extremely low limits of detection. Additional sensitivity gains are achieved by downscaling the chromatography system (as demonstrated with capillary LC [24], nano LC [21] and open tubular LC [25]. Regarding BCa tumours, a goal is to use as little sample as possible to allow multiple analysis of the same tumours; both derivatization and LC down-scaling are attractive tools in this regard. Also, in contrast to published GC-MS methods, LC-MS methods with derivatization allow for both free oxysterol determination (derivatization without hydrolysis) and total oxysterol determination (free + esterified and sulfonated oxysterols).

Separation of oxysterol isomers using conventional reversed phase LC particles/stationary phases can be challenging and time-consuming [19]. When samples take upwards of tens of minutes per sample then the high-throughput analyses required in large cohorts is not possible. However, with improvements in column particle technology (e.g. core-shell particles) and alternative stationary phases, the chromatographic process can be speeded up [19]. Shorter times have been achieved previously. For example Pataj et al. [20] have resolved oxysterol isomers using core-shell particles and a biphenyl phase in less than 8 minutes whilst McDonald et al. [26] have resolved side-chain oxysterols in less than 12 minutes using core-shell particles and C18 phase. With this in mind, we sought out to combine Girard T derivatization, core-shell particles, alternative stationary phases and downscaled LC for oxysterol determinations. We have combined these features with rugged on-line solid phase extraction [27, 28] to reduce manual sample preparation. The final method was applied to tumour samples from 22 BCa patients to examine and compare the endogenous concentration of free and total scOHCs in ER-positive and ER-negative primary breast tumours. Tumour heterogeneity was examined by cutting tumours into multiple aliquots.

2. Materials and methods

2.1 Chemicals and solutions

All reagents were of HPLC grade or higher. Methanol (MeOH) and acetonitrile (ACN) were purchased from VWR (Radnor, PA, USA) and were used to make mobile phases together with formic acid (98 %, Sigma-Aldrich, St. Louis, MO, USA) and type 1 water from a Millipore Milli-Q ® type 1 ultrapure water system (Merck KGaA, Darmstadt, Germany).

24S-Hydroxycholesterol, 25-hydroxycholesterol, 22R-hydroxycholesterol, 27-hydroxycholesterol (also known as 25R,26-hydroxycholesterol), 25-hydroxycholesterol-d_6, 27-hydroxycholesterol-d_6 and 22R-hydroxycholesterol-d_7 were purchased from Avanti Polar Lipids (Alabaster, AL, USA) . Stock solutions (100 µM) were prepared in nitrogen flushed ethanol and diluted to 1 nM (standard mixture of 22R-OHC, 24S-OHC, 25-OHC and 27-OHC) or 1.5 nM (deuterated internal standard mixture of 22R-OHC-d7, 25-OHC-d6 and 27-OHC-d6) with 2-propanol (WVR). Aliquots of 100 µL internal standard mixture and 37 - 370 µL standard mixture were used to make calibration solutions in the concentration range of 50 - 500 pM (200 pM internal standards) as described in [21]. The calibration solutions were based on methanol due to lack of appropriate matrices not containing endogenous oxysterols. Pooled plasma samples (5 µL) spiked with 37-370 µL standard mixture and 100 µL internal standard mixture was used to evaluate the method in the concentration range of 50 -500 pM (200 pM internal standards). Spiked pooled plasma samples were applied to an Oasis PRiME HBL 1 cc (30 mg) SPE cartridge (Waters, Milford, MA, USA) and eluted with 200 µL MeOH followed by sample preparation as described below for BCa tumour samples.

2.2 Derivatization with Girard T reagent

Analytes were “charge-tagged” following the procedure described by Griffiths and co-workers [29] with modifications as described in [21]. Briefly, aliquots of 200 µL 0.03 mg/mL cholesterol oxidase from *Streptomyces sp.* (Sigma-Aldrich) in 50 mM phosphate buffer pH 7 were added and the solutions were heated to 37 °C for one hour. To each sample/standard solution 15 mg Girard T reagent (Sigma-Aldrich), 15 µL glacial acetic acid (VWR) and 500 µL MeOH were added and reaction took place overnight, in the dark at room temperature. All solutions were stored at 4 °C after derivatization.

2.3 Breast cancer tumour samples

Breast cancer tumours were obtained from the Leeds Breast Research Tissue Bank (IRAS ID: 170113; Tissue Access Committee approval: LBTB_TAC_1/17). Tumours from ER-positive (n=11) and ER-negative (n=11) BCa patients were used in this study (Table 1). For more patient characteristics see *Supplementary material table S1*. Three consecutive slices from each tumour (approx. 5 mg per slice)

were collected and homogenized separately in 500 µL 1.5 nM internal standard mixture solution and 30 µL autoxidation monitoring solution (6 µM cholesterol-25,26,27-13C, Sigma-Aldrich) using an IKA T10 Ultra-Turrax homogenizer (VWR). When sample size was ample, the slices were collected from the middle of the tumours, and not the edges. For free oxysterols analysis, 100 µL sample solution was mixed with 100 µL 2-propanol and applied to a Oasis PRiME HLB 1 cc (30 mg) SPE cartridge (Waters) and the oxysterols were eluted with 200 µL MeOH. Solvents were evaporated in an Eppendorf concentrator plus (Hamburg, Germany) and residues were reconstituted in 20 µL 2-propanol followed by derivatization as described above. For total oxysterol (free and esterified) analysis, alkaline hydrolysis was performed by adding 35 µL 2 M KOH (Sigma Aldrich) in MeOH to 100 µL sample solution. Samples were heated at 60 °C for 120 min followed by liquid-liquid extraction (LLE) with n-hexane. To get phase separation, 150 µL type 1 water was added to the sample followed by 150 µL n-hexane (VWR). The samples were vortexed for 1 minute and centrifuged at 3000 rpm for 2.5 minutes. Hexane layer was removed, and the LLE was repeated twice with 150 µL n-hexane (combining all the hexane phases in the end). Solvents were evaporated in an Eppendorf concentrator plus and reconstituted in 200 µL 2-propanol. Samples were applied to an Oasis PRiME HBL 1 cc (30 mg) SPE cartridge and eluted off with 200 µL MeOH. Solvent was evaporated in an Eppendorf concentrator plus and samples reconstituted in 20 µL 2-propanol followed by derivatization as described above. Sample preparation was performed with and without cholesterol oxidase on one slice from each tumour to identify if any natural occurring 3-keto groups could be present above the detection level.

2.4 Chromatographic system

A Dionex UltiMate 3000 UHPLC system was connected to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltman, MS, USA) operated in selected reaction mode (SRM). For MS parameters see *supplementary material table S2*. Injection volume was 0.7 µL for column evaluation and increased to 60 µL when the AFFL-SPE-LC system was used (described below). All connections were Thermo Scientific Vipers™ stainless steel fingertight fittings (180 µm inner diameter (ID)). Columns investigated were ACE SuperPhenyl Hexyl (2.1 mm ID x 150 mm, d_p 2.5 µm, core-shell), ACE SuperC18 core-shell (2.1 mm ID x 150 mm d_p 2.5 µm, core-shell) (both from Advanced chromatography technologies LTD, Aberdeen, UK), Thermo Scientific HyperSil GOLD C18 (1 mm ID x 50 mm, d_p 1.9 µm), Waters Torus 2-PIC (2-picolylamine, 2.1 mm ID x 100 mm, d_p 1.7 µm) and Waters Torus 1-AA (1-aminoantracene, 2.1 mm ID x 100 mm, d_p 1.7 µm). In-house packed 0.1 mm ID x 100 mm columns using the ACE SuperPhenyl Hexyl particles was also investigated.

2.5 Automatic filtration and filter back flush solid phase extraction

A 10 port valve (Waters CapLC selector valve) with a 1 µm in-line filter and an on-line HotSEP C18 SPE column (Teknolab, Ski, Norway) was connected and controlled by the pump as shown in **Figure 1C**. Loading mobile phase was 0.1 % FA in type 1 H_2O delivered by a Hitachi L-7110 pump (Merck) with a flow rate of 500 µL/min. In position 1, solutions and samples were injected and filtered. Derivatized oxysterols were retained on the SPE column, while excess derivatization reagent was washed to waste. When the valve changed to position 2, the derivatized analytes are eluted off the SPE column and subsequently separated on an ACE SuperPhenyl Hexyl column (2.1 mm ID x 100 mm, core-shell) by a Dionex Ultimate 3000 UHPLC pump with a flow rate of 650 µL/min. Mobile phase A was 0.1 % FA in type 1 H_2O, B was 0.1 % FA in MeOH and C was 0.1 % FA in ACN. Isocratic elution conditions were 57/10/33 (v/v/v, A/B/C) for 4.3 minutes, followed by a washing step (50/50, v/v, B/C) for 2 minutes. Total method run time, including on-line sample clean-up and conditioning of the column was 8 minutes.

3. Results

3.1 Fast LC: Choice of stationary phase

Various types of reversed phase stationary phases were examined and compared to our previous used ACE C18 column (3 µm porous particles), with an overall goal to achieve baseline LC separations (Rs > 1.5) of 22R-OHC, 25-OHC, 24S-OHC and 27-OHC within 5 minutes. For each column, the mobile phase composition was optimized with regards to ACN and MeOH content. Good resolution was achieved using the Hypersil Gold column C18 (Rs = 1.4 – 4.2) by decreasing the column temperature to 15 °C. However, the analysis time was not considered to be acceptable (13 min). An ACE SuperC18 core-shell column provided shorter retention times, but not sufficient resolution (Rs = 0.8-5). Two more "exotic" stationary phases, namely Torous 2-PIC and Torous 1-AA, did not provide acceptable liquid chromatography of the oxysterols, resulting in either several minute-broad peaks (Torous 1-AA) and/or no separation of the oxysterol isomers (Torous 2-PIC). The best performing column was the ACE SuperPhenyl Hexyl column (core-shell, 2.1 mm ID x 150 mm) using a mobile phase consisting of a mixture of ACN, MeOH and water, together with a high flow rate (900 µL/min) and temperature (45 °C). All the Girard derivatized side-chain oxysterols were separated with R_s > 1.5 in 4 minutes (**Figure 1A**), meeting our above-mentioned goal.

3.2 Fast LC coupled with an on-line sample clean-up system

To remove excess derivatization reagent (which can e.g. contaminate the LC-MS system), we wanted to include the ACE SuperPhenyl Hexyl column in an automatic filtration and filter back-flush solid phase extraction (AFFL-SPE) system (**Figure 1C**). SPE columns with ACE SuperPhenyl Hexyl material was not commercially available, hence a standard C_{18} SPE column was used instead. Incorporation of the AFFL-SPE system allowed for robust reagent removal and analyte enrichment, but also resulted in some loss of resolution (R_s = 1.1-1.8) however still considered sufficient for quantitation. The total method run time was 8 min, including sample clean-up, separation, and washing/conditioning of the column. With this method, we could inject up to 60 µL of sample, resulting in calculated quantification limits (cLOQ) of > 31 pM, without chromatography deterioration (**Figure 1B**). With larger injection volumes, an interfering peak began to co-elute with 27-OHC (data not shown).

Narrower columns of 0.1 mm ID and 0.3 mm ID were also explored. In experiments with commercial and self-packed columns, the resolution of these columns was "under-par" compared to the larger 2.1 mm ID columns used above (data not shown). However, the 2.1. mm ID columns set-up described above was found to have sufficient sensitivity for our present task, as described below.

3.3 Method evaluation

The method was evaluated in the range of 50- 500 pM using pooled plasma samples (biologically complex, but more available compared to limited tumours samples which was the final matrix), see *supplementary material table S3.* The limit of quantification was calculated (cLOQ) from linearity curves and was in the range of 15 pM-31 pM. Apparent recovery was acceptable, in the range from 80 – 97 %. Intra-day precision was below 20 % for all concentration levels. Inter-day precision (calculated using single factor ANOVA) was between 10-23 % at lower LOQ (LLOQ), 5-10 % at medium LOQ (MLOQ) and 5 – 11 % at high LOQ (HLOQ). No carry-over was observed.

3.4 Oxysterol heterogeneity in breast cancer tumours

Free and total scOHCs in three separate slices of 11 ER-positive and 11 ER-negative tumours were determined. By analyzing three consecutive slices (5 mg) of each tumour sample revealed a large inter-tumour variability (RSD > 20 %) in scOHC concentrations. This variability was not always uniform across all target analytes, as some scOHCs were similar across intra-tumour triplicates, whilst others showed higher variability, even within the same tumours. While some samples showed minor concentration variations (**Figure 2B and C**), other tumours had alterations as to which scOHC had the highest concentration (**Figure 2A**, cut 1 and 2 have higher concentration of 27-OHC, while cut 3 has a higher

concentration of 24S-OHC). In general, this high inter-tumour variability meant there was extensive overlap in scOHCs concentration between all individual tumours irrespective of ER subtype status for both total and free scOHC (**Table 2**). 27-OHC was the most abundant OHC measured. Interestingly, in ER-positive tumours we observed a strong correlation between free and esterified 27-OHC (R^2=0.89, p<0.0001) and 25-OHC (R^2=0.82, p<0.01) but in ER-negative tumours these correlations were weak (27-OHC; R^2=0.61, p<0.05) or absent (24S-OHC and 25-OHC) (Figure 3).

4. Discussion

4.1 Fast LC of derivatized oxysterols

Sufficient sensitivity and resolution of the target oxysterols were achieved with the fast LC set-up using 2.1 mm ID column coupled in the on-line AFFL sample clean up system. Some resolution was lost compared to direct injection on the columns. Possible reasons for a loss of resolution may be additional extra-column contributions to band broadening, or that an ACE SuperPhenyl Hexyl trap column was not available, so a conventional C18 trap column was used instead. Hence, sufficient refocusing of the oxysterols on the column was not achieved due to larger relative retention on the SPE column compared to that on the separation column.

A fast separation (8 minutes including sample clean-up) was possible with high flow rate and temperature and core-shell particles. With core-shell particles a higher flow rate is possible without increased band broadening compared to that of fully porous particles. This is mainly due to decreased Eddy dispersion (A in Van Deemter equation), not lower resistant to mass transfer (C/u in Van Deemter equation) [30]. By using the SuperPhenyl Hexyl column, we did not observe any peak splitting of the derivatized oxysterols, which usually is observed for the Girard P/T hydrazone derivates [21, 22]. In addition to the analytes, several other peaks of unknown identity were observed (see e.g. early eluting peaks in 2B); this serves as an example of the need for chromatography, as non-separating interferences with similar MS features could have interfered with identification/quantification under non-optimized conditions.

Downscaling the chromatographic system (e.g. to 0.1 mm ID column) to achieve better sensitivity, hence use smaller sample size was not achieved as commercial columns in these dimensions are not available, and the in-house packed columns lacked efficiency. Although we may be able to optimize packing procedures for the ACE SPH solid core particles, reduced column efficiency of solid core particles in narrow ID is not too surprising [31]. Nevertheless, the 2.1 mm ID column with large volume injection (60 µL) was sufficiently sensitive to allow determination of BCa related oxysterols in 5 mg

tumour tissue. For some tumours, the sensitivity was too good and dilution of the samples (10 x) was performed.

4.2 Total vs. free oxysterols.

Oxysterols can be present both in their free from, but also as sulfonated and esterified versions in our body. In these samples, the measured oxysterols after alkaline hydrolysis are most likely to be free and esterified sterols, not sulfonated, due to lack of expression of the SULT2B1 enzyme in ER- BCa tumours (Data not published) The esterified version is connected to lipid storage and transport . Historically, GC-methods for oxysterols analysis has mostly been quantifying total oxysterols (e.g. after alkaline hydrolysis), whilste LC-MS method have mostly been quantifying free oxysterols. This has often led to confusion and trouble when comparing results and concentration levels of the different oxysterols in e.g. plasma (see [18] for table with comparison of free and total oxysterols in plasma samples). Hence, we wanted to compare the two methods, by splitting each sample cut in two (after homogenization) and subject one part for alkaline hydrolysis before derivatization into Girard T derivates. As expected, the total oxysterol concentrations were higher than the free oxysterols in the samples.

4.3 Oxysterol heterogeneity in breast cancer tumour

The huge intra tumour variation makes comparison of oxysterol concentration challenging. The reason for variation cannot be addressed to sample preparation, e.g. extraction efficiency, as the variation is both in absolute and relative concentration of the oxysterols. Changes in extraction efficiency of the oxysterols from the tumours should only yield changes in absolute concentration, hence the variation are reflecting tumour heterogeneity. The variations could possibly be due to differences in tumour invasion of oxysterol synthesizing macrophages, fibroblast and/or adipocytes. We could however observe a strong correlation between free and esterified 27-OHC and 25-OHC in ER-positive tumours suggesting intact oxysterol metabolism pathways in these tumours. The loss of correlation in ER-negative suggests alternative usage of esterified or free oxysterols in this more aggressive breast cancer type. 27-OHC is a selective estrogen receptor modulator and is linked to metastasis and proliferation of Estrogen Receptor positive BCa. 27-OHC can also activate the LXR receptor, a possible key player in metastatic and/or chemotherapy resistant triple negative BCa. Currently the relative contributions of free and esterified sidechain oxysterols to LXR signalling in breast cancer remains unexplored. The oxysterol heterogeneity of tumours is a critical factor when assessing the role of these lipids in cancer.

5. Conclusion

A fast LC-MS method for determination Girard T derivatized oxysterols was developed to examine BCa related oxysterols in tumours from both ER-positive and ER-negative patients. With large volume injection and automatic sample clean-up (AFFL) oxysterols could successfully be quantified from 5 mg sample size with a total LC-MS analysis time of 8 minutes per sample. Both free and total (after alkaline hydrolysis) scOHS concentration were examined. Multiple slices from same tumours revealed huge intra-tumour variations, hence revealing oxysterols heterogeneity and making comparisons challenging. Future studies could include assessing plasma vs. tumour levels, and expanding the method to measure other LXR-active sterols, e.g. 24S,25-epoxycholesterol.

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Figure 1. Extracted ion chromatogram (*m/z* 514.4 → 455.4) of ScOHC separated on an ACE SuperPhenyl Hexyl (2.1 mm ID x 150 mm column). **A**) Injection of 0.7 µL 2.8 µg/mL standard solution. Mobile phase was 0.1 % FA in H_2O/MeOH/ACN 60/8.5/31.5, v/v/v with a flow rate of 900 µL/min. Column temperature was 45 °C. R_S > 1.5. **B**) Injection of 50 pM standard solution using AFFL-SPE system. Mobile phase was 0.1 % FA in H_2O/MeOH/ACN, 57/10/33, v/v/v with a flow rate of 650 µL/min and column temperature 55 °C. R_S 1.1-1.8. **C**) Automatic filtration and filter backflush solid phase extraction (AFFL-SPE) LC-MS system. In load position, sample/standard solution (60 µL) is loaded (0.1 % FA in H_2O, 500 µL/min) through a filter, where particles are stopped. Oxysterols are retained on a Hotsep Kromasil C18 SPE column. Excess derivatization reagent is washed out to waste. After 30 seconds the valve is automatically switched to inject position, where oxysterols are eluted from the SPE and onto the ACE SuperPhenylHexyl column for separation. Simultaneously, particles on the filter are removed by back-flushing using 0.1 % FA in H_2O

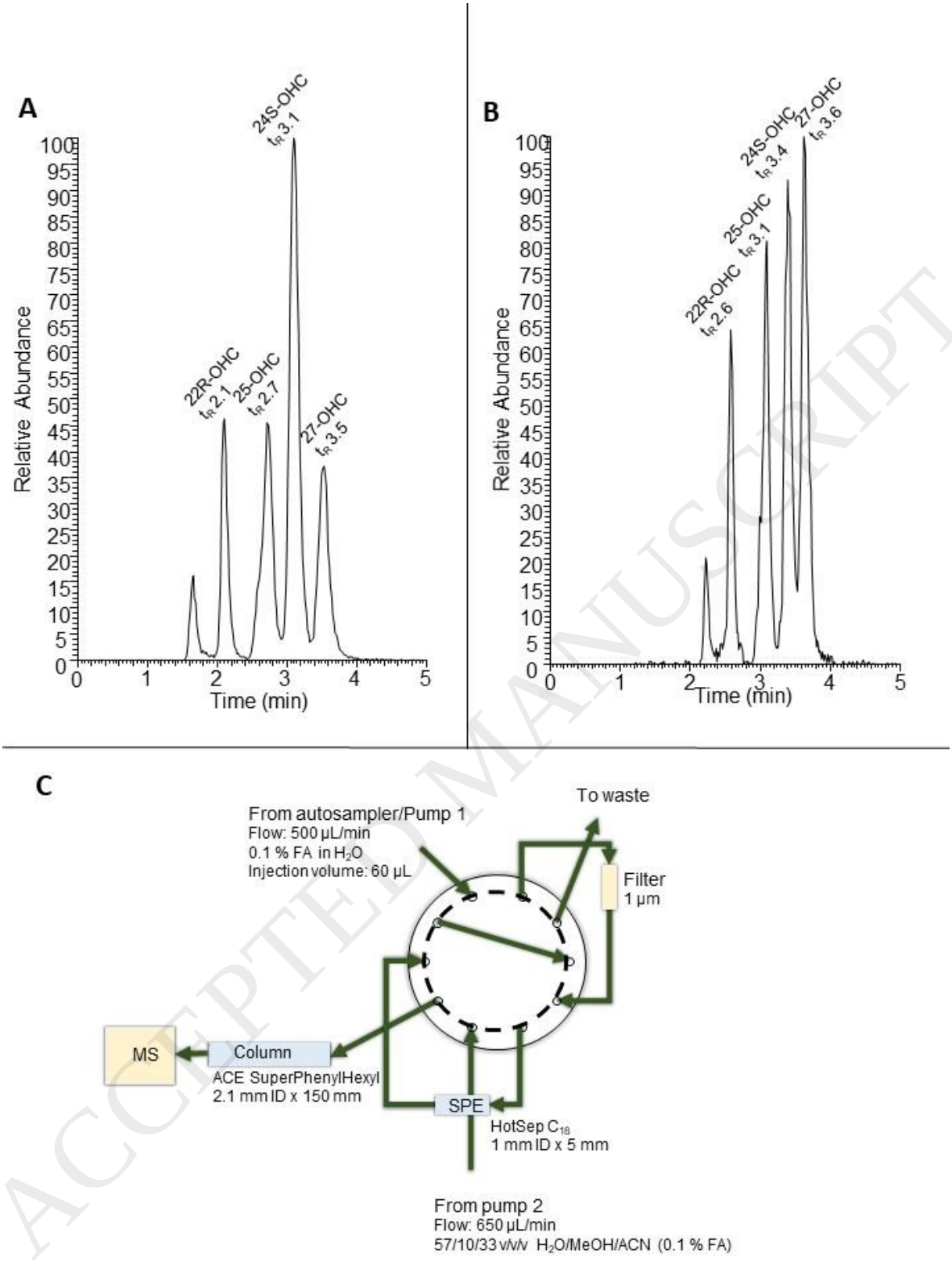

Figure 2. Intra-tumour oxysterol heterogeneity in breast cancer tumour. Extracted ion chromatogram (*m/z* 514.4 → 455.4) and quantification of ScOHC in different slices from breast cancer tumour. Both large (A) and minor (B, C) intra-tumour variation observed. Peak 1, 2 and 3 corresponds to 25-OHC, 24S-OHC and 27-OHC, respectively.

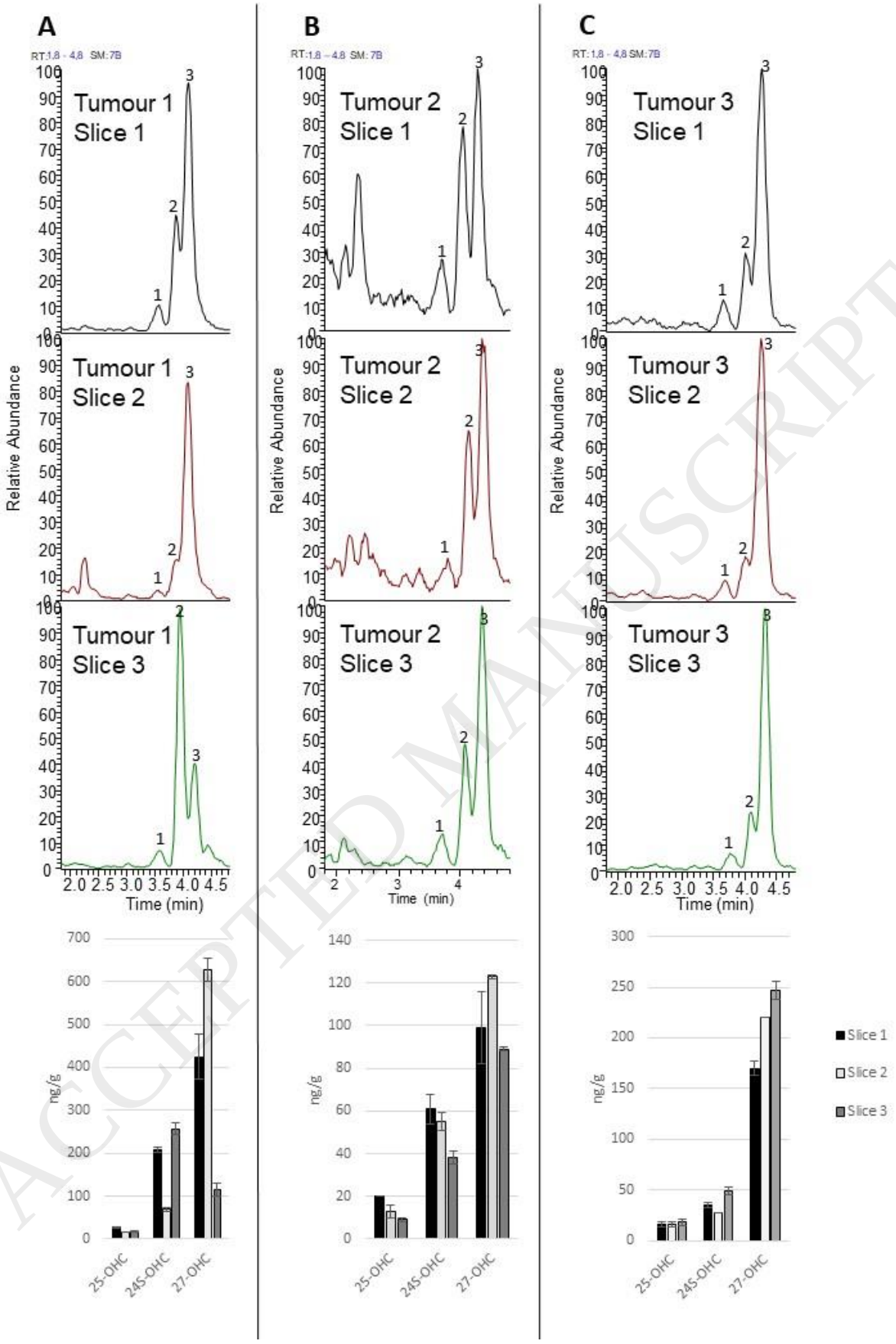

Figure 3. The concentration of free and esterified sidechain-OHC in patient BCa tumours correlate in ER- and ER+ subtypes. The oxysterol concentrations (total, esterified and free) in 22 patient tumours of two BCa subtypes (ER-positive n=11, ER-negative n=11) were measured by LCMS/MS and compared across subtypes. Free and esterified scOHC concentrations were analysed by Pearson correlation and assessed for statistical significance.

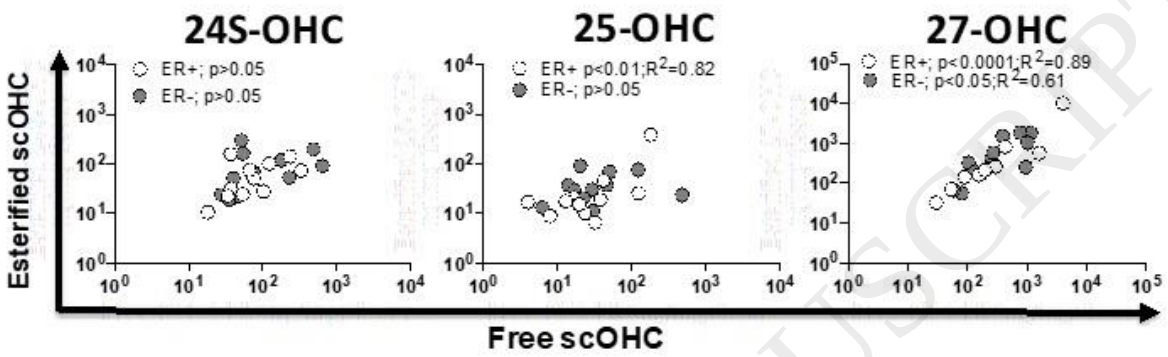

Table 1. Patient characteristics. Tumour tissue was obtained from the Leeds Breast Research Tissue Bank at Leeds Teaching Hospital Trust. *denotes tumour size not available for one patient.

Characteristic	Categories	No. of patients (%) n=22
ER Status	Negative	11 (50)
	Positive	11 (50)
PR Status	Negative	13 (59.1)
	Positive	9 (40.9)
HER2 Status	Negative	20 (90.9)
	Positive	2 (9.1)
Invasive Tumour Grade	1	2 (9.1)
	2	8 (36.4)
	3	12 (54.5)
Invasive Tumour Size*	</= 35mm	14 (63.6)
	>35mm	7 (31.8)
Survival Status	Alive	19 (86.4)
	Deceased	3 (13.6)
Recurrence/Metastasis	None	13 (59.1)
	Local and/or Distal	9 (40.9)

Table 2. Side-chain OHC concentrations in breast tumour samples. Total, esterified and free oxysterols were measured in three slices from each of 22 tumours (ER-positive n=11; ER-negative n=11). The minimum, maximum and mean concentrations are shown. There were no significant differences in oxysterol concentrations when comparing ER-positive and ER-negative tumours.

Side chain-OHC			ER-negative (ng/mg)	ER-positive (ng/mg)
24S-OHC	Total	Min	34	26
		Max	709	845
		Mean	229.73	165.06
	Ester	Min	3	10
		Max	390	361
		Mean	101.81	65.90
	Free	Min	18	16
		Max	973	484
		Mean	144.77	98
25-OHC	Total	Min	18	10
		Max	262	780
		Mean	76.96	88.96
	Ester	Min	3	2
		Max	135	476
		Mean	40	52.21
	Free	Min	4	3
		Max	165	304
		Mean	36.34	45.60
27-OHC	Total	Min	74	57
		Max	3732	2696
		Mean	908.53	438.92
	Ester	Min	8	23
		Max	3104	1015
		Mean	518.39	214.33
	Free	Min	46	24

	Max	1068	1942
	Mean	369.23	224.59